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Applicant(s): Takahide NISHI et al

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DECLARATION UNDER 37 CFR 1.132

I, KIYOSHI MORIMOTO, do hereby make the following declaration:

I am a co-inventor of the invention described and claimed in the above-identified application.

The U.S. Patent Examiner has raised a question as to whether some representative compounds encompassed by the instant compounds for Formula (I) where actually found to be antagonists at NK_1 and NK_3 receptors. Also the question of whether hyperactivity of NK_1, NK_2, and NK_3 receptors is implicated in the etiology of every known respiratory disease, allergic disease and urinary incontinence. These and other similar issues are raised with respect to the operability of the claimed compounds for the claimed purposes.

The following additional testing was accomplished under the Declarant's supervision and control, to provide further evidence of the statements of operability that were made in the specification and are claimed.

Presented below are the test results of testing disclosed compounds following test examples described in the specification. These tests are Test Example 1 (NK_1 receptor binding test) described at pages 185-186; Test Example 2 (NK_2 receptor binding test) described at pages 187-188; and Test Example 5 (NK_3 receptor binding test) described at pages 190-191.

For Test Example 1, the concentration of the compound was 100 ng/ml. For test Examples 2 and 5, the concentration of the compound was 10 ng/ml.

The compounds tested are the compounds described in the specification as Example 6 (pages 94-95), Examples 31-33 (pages 115-116); Example 58 (pages 137); Example 64 (page 143) and Example 69 (page 154).

The results of the tests are provided in the following tables:

Test results of the Test Example 1.

Test Substance	Inhibition rate (%)
Example 6	50.9
Example 31	69.0
Example 32	83.6
Example 33	64.7
Example 58	74.2
Example 64	67.8
Example 69	62.4

Test results of the Test Example 2.

Test Substance	Inhibition rate (%)
Example 6	45.1
Example 31	50.6
Example 32	57.5
Example 33	60.2
Example 58	65.1
Example 64	70.8
Example 69	53.0

Test results of the Test Example 5.

Test Substance	Inhibition rate (%)

Example 6	49.6
Example 31	89.6
Example 32	102.1
Example 33	85.5
Example 58	64.2
Example 64	58.4
Example 69	62.6

The method of computing Inhibition rate (%) of the Test Examples 1 and 2 refers to the discussion for Test Example 5 (see page 191 of the specification).

Concerning the relationship between hyperactivity of NK_1, NK_2, and NK_3 receptors to the etiology of the claimed ailments to be treated, the following is noted:

There is teaching or guidance present in Am Rev Respir Dis 1991 144 1187-1198, Am Rev Respir Dis 1991 144 1391-1399 and Life Sci. 2005 Jan 7;76(8):835-62 that hyperactivity of NK_1, NK_2 and NK_3 receptors is implicated in the etiology of every known respiratory disease, allergic disease and urinary incontinence.

Enclosed are copies of the Am Rev. Respir Dis 1991 144 1187-1198, Am Rev Respir Dis 1991 144 1391-1399 and Life Sci. 2005 Jan 7;76(8):835-62 (12 pages, 9 pages and 28 pages).

The respiratory diseases such as asthma, bronchitis and chronic obstructive lung disease are disclosed in Am Rev Respir Dis 1991 144 1187-1198 and Am Rev Respir Dis 1991 144 1391-1399.

The allergic diseases such as rhinitis are disclosed in Am

Rev Respir Dis 1991 144 1187-1198 and Am Rev Respir Dis 1991 144 1391-1399.

The urinary incontinence is disclosed in Life Sci. 2005 Jan 7;76(8):835-62.

It is submitted that evidence that the compounds of this patent application have prophylactic or therapeutic effect on the respiratory disease, allergic disease and urinary incontinence is in the combination of the test results of the Test Example 1, 2, and 5 and the contents of the above 3 references.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: OCT 1, 2007

Kiyoshi Morimoto

Kiyoshi Morimoto

Attachment: Am Rev Respir Dis 1991(1187-1198)
Am Rev Respir Dis 1991 (1391-1399)
Life Sci. 2005(835-62)

State of the Art

Neuropeptides in the Respiratory Tract

Part I[1,2]

PETER J. BARNES, JAMES N. BARANIUK, and MARIA G. BELVISI

Contents

Introduction

A diverse collection of neuropeptides are released from sensory, parasympathetic, and sympathetic neurons in the respiratory tract (table 1). These peptides have potent effects on bronchomotor tone, airway secretions, the bronchial circulation, and on inflammatory and immune cells (table 2). The precise physiologic roles of each peptide is still not known, but some clues are provided by their localization and functional effects (1–5). The purpose of this review is to discuss what is known of these peptides, particularly in the human respiratory tract, and to speculate on their possible pathophysiologic role in airway disease.

NANC Nerves

In addition to classic cholinergic and adrenergic innervation of airways, there are neural mechanisms that are not blocked by cholinergic or adrenergic antagonists (6–8). Nonadrenergic noncholinergic (NANC) nerves were first described in the gut, therefore their existence in the respiratory tract is to be expected. NANC nerves were initially conceived as a "third" nervous system in the lungs (9), but it rapidly became apparent that several different neural mechanisms are included. NANC mechanisms result in both bronchodilation and bronchoconstriction, vasodilation and vasoconstriction, and mucus secretion, indicating that several types of neurotransmitters may be involved.

Inhibitory NANC (i-NANC) nerves relax airway smooth muscle. They have been demonstrated *in vitro* by electrical field stimulation after adrenergic and cholinergic blockade in several species including humans (7, 8, 10). In human airway smooth muscle, the NANC inhibitory system is the only neural bronchodilator pathway, since there is no functional sympathetic innervation. Because NANC innervation is the sole inhibitory pathway from trachea to the smallest bronchi, there has been considerable interest in the identity of the neurotransmitter. Current evidence suggests that the neuropeptide vasoactive intestinal peptide (VIP) may mediate i-NANC effects in some species but that nitric oxide (NO) is also involved (11, 12). In human airways the predominant i-NANC transmitter appears to be NO (13).

Electrical stimulation of guinea-pig bronchi, and occasionally trachea *in vitro*, and vagus nerve *in vivo* produces a component of bronchoconstriction that is not inhibited by atropine (14). This bronchoconstrictor response has been termed the excitatory NANC (e-NANC) response. There is now convincing evidence that tachykinins released retrogradely from a certain population of sensory nerves mediate e-NANC responses. A similar e-NANC response has occasionally been reported in human airways *in vitro*, but this is not consistent (14).

Other NANC responses have been described in airways, NANC-mediated secretion of mucus has been demonstrated in cats *in vivo* using vagal nerve stimulation (15) and in ferret airways *in vitro*, using electrical field stimulation (16). NANC regulation of airway blood flow has been demonstrated in several species (17, 18).

Cotransmission

Although NANC nerves were originally envisaged as an anatomically separate nervous system, it is now more likely that NANC neural effects are mediated by the release of neurotransmitters from classic autonomic nerves. Thus the i-NANC responses in airway smooth muscle are likely to be mediated by the release of cotransmitters such as NO and VIP from cholinergic nerves (figure 1). NANC vasoconstrictor responses are mediated by the release of neuropeptide Y (NPY) from adrenergic nerves, whereas e-NANC bronchoconstrictor responses are mediated by the release of tachykinins from unmyelinated sensory nerves. The physiologic relevance of cotransmission is

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This is Part 1 of two parts; Part 2 will appear in the next issue of the Review.

TABLE 1

NEUROPEPTIDES IN THE RESPIRATORY TRACT

Peptide	Localization
Vasoactive intestinal peptide	Parasympathetic
Peptide histidine isoleucine/ methionine	Parasympathetic
Peptide histidine valine-42	Parasympathetic
Helodermin	Parasympathetic
PACAP-27	Parasympathetic
Galanin	Parasympathetic
Substance P	Afferent
Neurokinin A	Afferent
Neuropeptide K	Afferent
Calcitonin gene-related peptide	Afferent
Gastrin-releasing peptide	Afferent
Neuropeptide Y	Sympathetic
Somatostatin	Afferent/uncertain
Enkephalin	Afferent/uncertain
Cholecystokinin octapeptide	Afferent/uncertain

Definition of abbreviations: PACAP = pituitary adenylate cyclase-activating peptide.

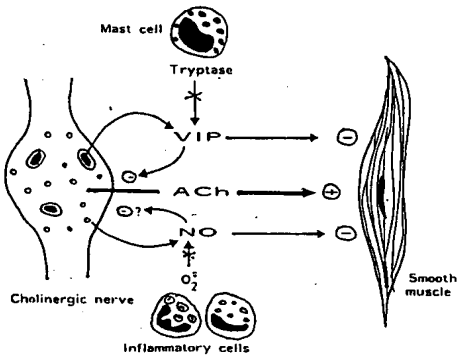

Fig. 1. VIP and nitric oxide (NO) may be coreleased from cholinergic nerves and act as functional antagonists of cholinergic bronchoconstriction. In addition they may act prejunctionally to inhibit ACh release. In asthma enzymes such as tryptase released from airway mast cells may rapidly degrade VIP, and oxygen free radicals, such as superoxide anions (O_2^-) from inflammatory cells may inactivate NO, thus leading to exaggerated cholinergic neural bronchoconstriction.

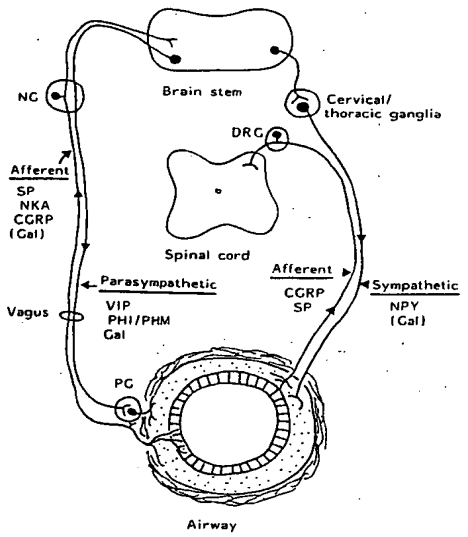

Fig. 2. Innervation of lower respiratory tract, showing neuropeptide colocalization in autonomic nerves. DRG = dorsal root ganglion; NG = nodose/jugular ganglion; PG = parasympathetic ganglion; SP = substance P; NKA = neurokinin A; CGRP = calcitonin gene-related peptide; Gal = galanin; VIP = vasoactive intestinal peptide; PHI = peptide histidine isoleucine; NPY = neuropeptide Y.

likely to be related to the "fine tuning" of classic autonomic nerves, but the role of the cotransmitters may become more apparent in disease (19).

Coexistence of several peptides within the same nerve is commonly described in the peripheral nervous system, and multiple combinations are possible, giving rise to the concept of "chemical coding" of nerve fibers. VIP and PHI usually coexist since they are derived from the same precursor peptide coded by a single gene. Galanin is often present with VIP in cholinergic neurons (20, 21). In sensory nerves substance P (SP), neurokinin A (NKA), and calcitonin gene-related peptide (GCRP) often coexist, but some sensory nerves may also contain galanin and VIP (22). Similarly, adrenergic nerves that contain NPY may also contain somatostatin, galanin, VIP, and enkephalin (23). Thus, there is a complex distribution of neuropeptides in the innervation of the upper and lower respiratory tracts, with the same peptides occurring in different types of nerves (figures 2 and 3). The physiologic significance of this complexity is not yet clear, but it seems likely that there may be functional interactions between the multiple neuropeptides released and the classic transmitters that allow complex integration and regulation of functions in the airway.

Neuropeptide Effects

Neuropeptides produce many different effects mediated by surface receptors, and the functional responses in the respiratory tract depend upon the localization of receptors and the second messengers linked to receptor activation in the target cells. In addition to acute effects, such as contraction and relaxation of airway smooth muscle, there may be chronic effects of neuropeptides such as effects on growth and development (24). Several neuropeptides, most notably gastrin releasing peptide and bombesin, have already been implicated in airway epithelial growth, and it is likely that the trophic effects of neuropeptides will be increasingly recognized.

Vasoactive Intestinal Peptide

VIP is a 28 amino acid peptide that was originally extracted from porcine duodenum as a vasodilator peptide (25). VIP is localized to several types of nerve in the respiratory tract of several species, including humans. VIP has potent effects on airway and pulmonary vascular tone and on airway secretion, which suggests that it may have an important regulatory role (26, 27).

Localization

VIP has been isolated from lung extracts of several species, including humans and is one of the most abundant of the neuropeptides found in lung (28) and nasal mucosa (29). VIP-immunoreactivity(IR) is localized to nerves and ganglia in airways and pulmonary vessels (1, 2, 30, 31). VIP-IR is present in ganglion cells in the posterior trachea and around intrapulmonary bronchi, diminishing in frequency as the airways become smaller. Usually VIP-IR neurons are found in parasympathetic ganglia, but isolated ganglion cells are also seen. These cells give rise to intrinsic VIP-ergic motor nerves that

TABLE 2

EFFECTS OF NEUROPEPTIDES ON AIRWAY FUNCTIONS

Peptide	ASM	Mucus secretion	Vessels	Nerves	Other cells
VIP*	Relax	Increase/Decrease	Dilate	↓ Chol/e-NANC	↓ Mast cells/T-lymphocytes
SP	(Contract)	Increase	Dilate ↑ Leak	(↑ Chol)	↑ Macrophage/monocytes
NKA	Contract	(Increase)	(Dilate)	↑ Chol	↑ Macrophage
CGRP	(Contract)	(Increase)	Dilate	?	↓ Macrophage
NPY	(Contract)	Increase	Constrict	↓ Chol/e-NANC	?
GRP	Contract	Increase	Constrict	?	↑ Epithelial growth
CCK_8	Contract	?	?	No effect	?
Galanin	No effect	?	No effect	↓ e-NANC	?

Definition of abbreviations: ASM = airway smooth muscle; VIP = vasoactive intestinal peptide; SP = substance P; NKA = neurokinin A; CGRP = calcitonin gene-related peptide; NPY = neuropeptide Y; GRP = gastrin-releasing peptide; CCK_8 = cholecystokinin; e-NANC = excitory nonadrenergic noncholinergic. Parentheses indicate small or uncertain effects.

* VIP-related peptides PHI/M, PACAP-27, and helodermin have similar effects.

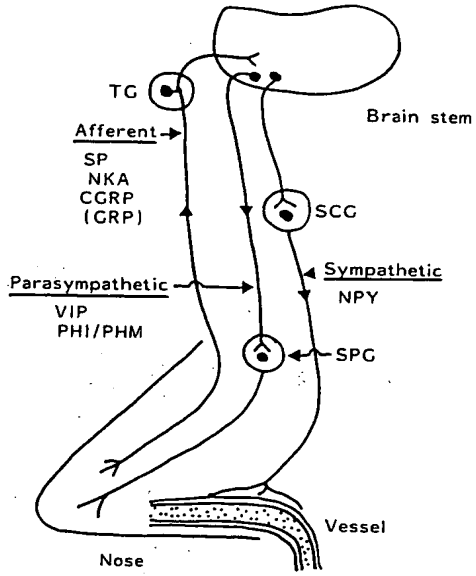

Fig. 3. Innervation of the nose showing colocalization of neuropeptides. TG = trigeminal ganglion; SCG = superior cervical ganglion; SPG = sphenopalatine ganglion.

are largely cholinergic. Several functional populations of ganglion cells are recognized (32), but the relationship between different ganglion cells and neuropeptide content is not yet known.

VIP-IR nerves are widely distributed throughout the respiratory tract and pulmonary vasculature. There is a rich VIP-ergic innervation of the nasal mucosa and upper respiratory tract (29) and in the proximal airways, but the density of innervation diminishes peripherally so that few VIP-ergic fibers are found in bronchioles (1, 2, 31). The pattern of distribution largely follows that of cholinergic nerves in airways consistent with the colocalization of VIP and acetylcholine in human airways (33). VIP-ergic nerves are found within airway smooth muscle, around bronchial vessels and surrounding submucosal glands. VIP may be localized to some sensory nerves, including subepithelial nerves in the airways, which may arise in the jugular and nodose ganglia (22, 31). VIP, at least in some species, may also be localized to sympathetic nerves (22). VIP-IR nerves are markedly depleted in older animals (34), which may have important clinical implications in terms of airway disease in the elderly. A striking depletion of VIP-IR nerves has been reported in patients with severe asthma (35), and reduced VIP-IR nerves are found in association with sweat glands of patients with cystic fibrosis (36).

Receptors

VIP receptors have been identified in the lung of several species by receptor binding techniques using [^{125}I]VIP (37, 38). Binding of VIP to its receptor activates adenylyl cyclase, and VIP stimulates cyclic AMP formation in lung fragments (39). The actions of VIP are, therefore, similar to those of β-adrenoceptor agonists, and any differences in response of different tissues to VIP or β-agonists depends on the relative densities or coupling of their respective receptors. The distribution of VIP receptors in lung has been investigated using an autoradiographic method to map out specific VIP binding sites (40). VIP receptors are found in high density in pulmonary vascular smooth muscle and in airway smooth muscle of large, but not small, airways. VIP receptors are also found in high density in airway epithelium and submucosal glands. VIP binding sites in human nasal mucosa are found on epithelial cells, submucosal glands, and arterial but not sinusoidal vessels (29). The distribution of receptors is consistent with the known functions of VIP.

A high density of VIP binding sites is also found in alveolar walls. The physiologic function of these receptors is obscure because there is no VIP-ergic innervation to peripheral lung. It is possible that these VIP binding sites represent sites of uptake of VIP because VIP is taken up from the circulation and metabolized by pulmonary capillary endothelial cells. The distribution of VIP receptors has also been studied by a functional immunocytochemical method using an antibody to cyclic AMP. After stimulation by VIP, cyclic AMP increases in those cells with specific response. This technique confirms the autoradiographic studies by demonstrating VIP receptors in airway smooth muscle, epithelium, and submucosal glands of several species (41).

Effects on Airway Smooth Muscle

VIP is a potent relaxant of airway smooth muscle *in vitro*, and this relaxation is independent of adrenergic receptors (26). VIP is more potent than isoproterenol in relaxing human bronchi, making it one of the most potent endogenous bronchodilators so far described (42). Because there is a rich VIP-ergic innervation of human bronchi, this suggests that VIP may be an important regulator of bronchial tone and may be involved in counteracting the bronchoconstriction of asthma. The response to VIP may depend on the size of the airway. In human airways, bronchi are potently relaxed by VIP, while bronchioles are unaffected. In contrast, both relax to an equal degree with isoproterenol (42). This response of human airways is consistent with the distribution of VIP-receptors because receptors are to be seen in bronchial smooth muscle but not in bronchiolar smooth muscle (40). This peripheral diminution of VIP-receptors is also consistent with the distribution of VIP-IR nerves that diminish markedly as airways become smaller (31). These studies suggest that VIP, while regulating the caliber of large airways, is unlikely to influence small airways.

Intravenously administered VIP causes bronchodilation in cats *in vivo* (43). Inhaled VIP protects against the bronchoconstrictor effects of histamine and prostaglandin $F_{2\alpha}$ in dogs (44). In asthmatic patients, however, inhaled VIP has no bronchodilator effect, although a β_2-adrenergic agonist in the same subjects is markedly effective (45). Inhaled VIP has only a small protective effect against the bronchoconstrictor effect of histamine (46) and has no effect against exercise-induced bronchoconstriction (47). This lack of potency of inhaled VIP may be explained by the epithelium, which possess several proteolytic enzymes and may present a barrier to diffusion. Infused VIP has no bronchodilator effect in normal subjects who readily bronchodilate with isoproterenol (46). However, infusion of VIP produces flushing, marked hypotension and reflex tachycardia. These effects limit the dose that can be given by infusion and, as VIP has a more potent relaxant effect on vessels than on airway smooth muscle, this prevents administration of a sufficient bronchodilating dose. Infused VIP causes bronchoconstriction in asthmatic subjects, but the effect is trivial (48) and might be explained by the reflex sympathoadrenal activation secondary to the profound cardiovascular effects. Thus, although VIP has potent bronchodilator effects *in vitro*, it has no significant action when inhaled *in vivo* and therefore has little therapeutic potential. More stable analogs or novel compounds that activate VIP-receptors would be unlikely to have any advantage over existing β_2-agonists.

Effects on Airway Secretion

VIP-IR nerves are closely associated with airway submucosal glands and form a dense network around the gland acini (1,

2, 30). VIP potently stimulates mucus secretion measured by ^{35}S-labeled glycoprotein secretion in ferret airway *in vitro*, being significantly more potent than isoproterenol (49). VIP increases cyclic AMP formation in submucosal gland cells, and there is some suggestion that, as with β-adrenergic agonists, there may be preferential effects on mucous rather than serous cells of these glands (41), indicating that VIP may stimulate a secretion rich in mucous glycoproteins in some species. VIP-receptors have been localized to human submucosal glands, suggesting that VIP-ergic nerves may regulate mucus secretion in human airways (40). VIP has an inhibitory effect on glycoprotein secretion from human tracheal explants (50), which is surprising because agonists that stimulate cyclic AMP formation would be expected to stimulate secretion. More recently the effects of VIP on mucus secretion have been found to be more complex and may depend on the drive to gland secretion. For example, mucus secretion stimulated by cholinergic agonists is inhibited in ferret trachea but stimulated in cat trachea, whereas secretion stimulated with the α-adrenergic agonist phenylephrine is augmented (51, 52). In contrast, VIP stimulates serous cell lactoferrin secretion from human nasal mucosal explants *in vitro* but has little effect on mucous glycoprotein release (29).

VIP is a potent stimulant of chloride ion transport and therefore water secretion in dog tracheal epithelium (53), suggesting that VIP may be a regulator of airway water secretion and therefore mucociliary clearance. The high density of VIP-receptors on epithelial cells of human airways suggests that VIP may regulate ion transport and other epithelial functions in human airways (40).

Vascular Effects

VIP is a potent dilator of systemic and pulmonary vessels. *In vitro* VIP potently relaxes pulmonary vessels in many species including humans (25, 54). The relaxation is independent of endothelial cells, indicating that VIP acts directly on vascular smooth muscle cells rather than by releasing a relaxant factor from endothelial cells (54, 55). This is confirmed by autoradiographic studies showing the high density of receptors in smooth muscle with no labeling of endothelial cells (40, 55). The density of VIP-receptors is significantly greater in human pulmonary vessels than on bronchial smooth muscle, which may explain why VIP is about tenfold more potent as a vasodilator than a bronchodilator *in vitro*.

VIP increases airways blood flow in dogs and pigs and is more potent on tracheal than on bronchial vessels (17, 18). There is convincing evidence that VIP is the mediator of NANC vasodilation in trachea, whereas in more peripheral airways other neuropeptides are involved (56). Because VIP is likely to have a greater effect on bronchial vessels than on airway smooth muscle, it may provide a mechanism for increasing blood flow to contracted smooth muscle. Thus, if VIP is released from cholinergic nerves, it may improve muscular perfusion during cholinergic contraction. Perhaps the small protective effect of inhaled VIP against histamine-induced bronchoconstriction in human subjects (57), despite a lack of effect on bronchomotor tone, may be explained by an increase in bronchial blood flow, which would more rapidly remove inhaled histamine from sites of deposition in the airways.

VIP is a powerful vasodilator of nasal vessels (58). VIP may account for the atropine-resistant vasodilatation that occurs on vidian nerve stimulation in feline nasal mucosa because a rise in VIP concentration in efferent blood is detectable (59). VIP released during parasympathetic reflexes may contribute to nasal congestion.

Neuromodulatory Effects

VIP is localized to nerves that surround airway ganglia, suggesting a possible neuromodulatory effect on cholinergic neurotransmission. VIP appears to modulate cholinergic neurotransmission in guinea pig parasympathetic ganglia (60) and postganglionic nerves (61–63), because it has a greater inhibitory effect on neurally-induced bronchoconstriction than on an equivalent contractile response induced by exogenous acetylcholine. VIP also modulates the release of peptides from sensory nerves in guinea pig bronchi *in vitro* (63).

Antiinflammatory Actions

VIP inhibits release of mediators from pulmonary mast cells (64) and may have several other antiinflammatory actions in airways. VIP may interact with T lymphocytes and has the potential to act as a local immunodulator in airways (65). VIP may protect the lung against HCl-induced pulmonary edema and may act as a free radical scavenger (66).

VIP as an i-NANC Neurotransmitter

Several lines of evidence implicate VIP as a neurotransmitter of i-NANC nerves in airways. VIP produces prolonged relaxation of airway smooth muscle that is unaffected by adrenergic or neural blockade and has a time-course similar to that of i-NANC response both *in vitro* and *in vivo* in several species. VIP mimics the electrophysiologic changes in airway smooth muscle produced by NANC nerve stimulation (67, 68). Electrical field stimulation of tracheobronchial preparations releases VIP into the bathing medium, and this release is blocked by tetrodotoxin, proving that it is derived from nerve stimulation (67, 69). Furthermore, the amount of VIP released is related to the magnitude of nerve stimulation. Several antagonists of VIP receptors have been developed, whereas these may weakly inhibit some actions of VIP, they do not inhibit the effects of VIP on airway smooth muscle (70). In the absence of potent specific blockers of VIP-receptors other strategies have been adopted. Incubation of cat and guinea-pig trachea with high concentration of VIP induces tachyphylaxis and also reduces the magnitude of NANC nerve relaxation, whereas responses to sympathetic nerve stimulation and isoproterenol are unaffected (68, 69, 71). VIP relaxes airway smooth muscle by increasing intracellular cyclic AMP, and its effects are therefore potentiated by a selective inhibitor of cyclic AMP phosphodiesterase (SK&F 94120), which normally degrades intracellular cyclic AMP (72). Under the same experimental conditions, this phosphodiesterase inhibitor also potentiates i-NANC responses in guinea-pig trachea, whereas an inhibitor of cyclic GMP phosphodiesterase (zaprinast) does not, supporting the idea that the i-NANC transmitter activates adenylyl cyclase. Perhaps the most convincing evidence that VIP is a transmitter of i-NANC nerves in airways are studies with enzymes that degrade this peptide. VIP is rapidly broken down into active fragments by trypsin and α-chymotrypsin and also by mast cell tryptase (73–75). Incubation of guinea-pig trachea with α-chymotrypsin, under conditions that completely block responses to exogenous VIP, results in a significant reduction in i-NANC response (76). However, inhibition is incomplete indicating that some other transmitter may be involved. A similar experimental design in feline airways shows no effect of α-chymotrypsin on i-NANC response (77), sug-

gesting that there may be differences in the i-NANC transmitter between species. In human tracheal strips, the i-NANC response is unaffected by α-chymotrypsin, but it is possible that the enzyme does not have good access to the sites where VIP is released (13). The close association between responses to VIP and NANC relaxation in different sizes of human airways (42) provides supportive evidence for VIP as a neurotransmitter.

Some evidence argues against VIP as a neurotransmitter of i-NANC in airways. After pretreatment of guinea-pig trachea with maximally effective concentrations of VIP, there is no diminution of i-NANC relaxation, which would be expected if all VIP-receptors were occupied (78). However, exogenous VIP may not have ready access to the VIP receptors related to VIP-ergic nerves. Removal of the epithelium potentiates the bronchodilator action of VIP *in vitro* but has no enhancing effect on i-NANC responses (79). This might be because VIP is released from cholinergic nerves distant from airway epithelium. In addition, there is convincing evidence from guinea-pig and human airways that NO contributes to bronchodilator i-NANC responses (11–13). The precise role of VIP as an i-NANC transmitter can only be resolved when potent and specific VIP antagonists become available.

Cotransmission with Acetylcholine

VIP coexists with acetylcholine (ACh) in some cholinergic nerves, supplying exocrine glands and potentiates the salivary secretory responses to ACh (80). VIP may be released from cholinergic nerves only with high frequency firing and may serve to increase the blood flow to exocrine glands under conditions of excessive stimulation. VIP also appears to coexist with ACh in airways (33), and it seems likely that there is a functional relationship between VIP and cholinergic neural control.

It is possible that excessive stimulation of cholinergic nerves and certain patterns of firing result in VIP release. In bovine tracheal smooth muscle, VIP has an inhibitory effect on cholinergic nerve-induced contraction only with high frequency firing, and also reduces the contractile effect of exogenous ACh (81). This does not involve any change in muscarinic receptor density or affinity, and may be due to functional antagonism. VIP also has an inhibitory effect on the release of ACh from airway cholinergic nerves (60–63). Conversely α-chymotrypsin, which degrades VIP, potentiates cholinergic nerve induced contractions in guinea-pig airways (82). VIP and NO seem to counteract the bronchoconstrictor effect of cholinergic bronchoconstriction and thus may function as a "braking" mechanism for airway cholinergic nerves (83) (figure 1). If this mechanism were to be deficient with either reduced release or increased breakdown of VIP, then an exaggerated bronchoconstrictor response may result.

Possible Abnormalities in Asthma

Whether dysfunction of VIP-ergic innervation contributes to airway disease is uncertain. A striking absence of VIP-IR nerves has been described in the lungs of patients with asthma in tissues largely obtained at postmortem (36). The loss of VIP-IR from all tissues including pulmonary vessels is so complete that it seems unlikely to represent a fundamental absence of VIP-IR nerves in asthma. More likely is the possibility that enzymes, such as mast cell tryptase, are released from inflammatory cells in asthma and that these rapidly degrade VIP when sections are cut (84). Biopsies taken from patients with mild asthma suggest that VIP-immunoreactive nerves appear normal in asthma (85). VIP antibodies, which would neutralize the effects of VIP, have also been described in the plasma of asthmatic patients (86). They are found with the same prevalence in nonasthmatic patients, so that their significance is doubtful. Although it seems unlikely that there would be any primary abnormality in VIP innervation in the airways of patients with asthma, it is possible that a secondary abnormality may arise as a result of the inflammatory process in the airway.

Mast cell tryptase is particularly active in degrading VIP (73, 75) and is known to be elevated in asthmatic airways (87). Inhibition of tryptase potentiates the bronchodilator response to VIP in human airways *in vitro* (88). Mast cell tryptase reverses the relaxation of airways induced by VIP (89) and markedly increases the *in vitro* responsiveness of canine airways to histamine (90). Tryptase released from mast cells in the asthmatic airway may then more rapidly degrade VIP and related peptides released from airway cholinergic nerves. This would remove a "brake" from cholinergic nerves and lead to exaggerated cholinergic reflex bronchoconstriction (figure 1). This may also have the effect of increasing inflammatory responses in the airway, because VIP has antiinflammatory actions. In addition NO may also be more rapidly degraded by oxidants, such as superoxide anion released from activated inflammatory cells, further adding to the increase in cholinergic tone and inflammatory effects.

Whether i-NANC responses are impaired in asthma is not yet certain. In patients with mild asthma no evidence for an impaired NANC bronchodilator reflex has been observed (91, 92). However, this does not preclude a defect in more severe asthmatics, in whom the degree of airway inflammation may be greater. In sensitized cats exposed to allergen, a reduction in i-NANC responses has been reported (93). This is presumably caused by the release of enzymes or oxygen free radicals from inflammatory cells in the airways. However, as discussed previously, the contribution of VIP to i-NANC responses in human airways is not yet established, and increased degradation of this peptide in asthma may have a relatively minor effect on airway tone.

VIP-related Peptides

Several other peptides have now been identified in the mammalian nervous system which are similar in structure and effect to VIP.

Peptide Histidine Isoleucine

Peptide histidine isoleucine (PHI) and its human equivalent peptide histidine methionine (PHM) have a marked structural similarity to VIP, with 50% amino acid sequence homology. PHI and PHM are encoded by the same gene as VIP and both peptides are synthesized in the same prohormone (94). It is therefore not surprising to find that PHI has a similar immunocytochemical distribution in lung to VIP and that PHI-IR nerves supply airway smooth muscle (especially larger airways), bronchial and pulmonary vessels, submucosal glands, and airway ganglia (23, 95, 96). The amount of PHI is very similar to the amount of VIP in respiratory tract (96). Like VIP, PHI stimulates adenylyl cyclase and appears to activate the same receptor as VIP (38).

There are some differences between VIP and PHI, because PHI is less potent as an airway vasodilator (18, 97) and more potent as a stimulant of secretion than VIP (98). Like VIP, PHI potentiates cholinergic and inhibits α-adrenergic stimulation of mucus secretion *in vitro* (98). In human bronchi *in vitro* PHM is a potent relaxant and is equipotent to VIP (42). It is likely that PHI/PHM is

released with VIP from airway nerves and may also be a contributory neurotransmitter in i-NANC nerves.

Peptide Histidine Valine

Peptide histidine valine (PHV-42) is an N-terminally extended precursor of VIP. PHV is a potent bronchodilator of guinea-pig airways *in vitro* (99), but when infused in asthmatic patients has no demonstrable bronchodilator effect (100). It is not yet clear whether this peptide is released from airway nerves.

Helodermin

Helodermin is a 35 amino acid peptide of similar structure to VIP, which has been isolated from the salivary gland venom of the Gila monster lizard. Helodermin-IR has been localized to airway nerves, and the peptide has similar effects to VIP but has a longer duration of action. Helodermin is a potent relaxant of airway smooth muscle *in vitro*, and helodermin-IR has been reported in trachea (101). Helodermin appears to activate a high affinity form of the VIP receptor (38).

Pituitary Adenylate Cyclase Activating Peptide

Pituitary adenylate cyclase activating peptide (PACAP), a 38 amino acid peptide isolated from sheep hypothalamus, and PACAP-27, a truncated fragment, have marked sequence homology with VIP and have been demonstrated in the peripheral nervous system (102). PACAP-IR has a similar distribution to VIP in airways of several species, and may be localized to cholinergic and also to capsaicin-sensitive afferent nerves (103). The effects of PACAP-27 are likely to be similar to those of VIP. There appears to be a particularly high density of receptors for PACAP in lung tissue (104).

Tachykinins

Although SP was isolated over 50 years ago, structurally-related peptides (tachykinins) called neurokinin A (NKA) and neurokinin B (NKB) have now been identified in the mammalian nervous system (105, 106). Tachykinins are a family of peptides with the common C-terminal sequence Phe-X-Gly-Leu-Met-NH_2. NKA and SP are coded by the same preprotachykinin (PPT) gene, which produces three mRNAs: α-PPT produces SP alone; β-PPT codes for SP, NKA, and its N-terminally extended form neuropeptide K (NPK); and gamma-PPT produces SP, NKA, and a novel N-terminally extended form of NKA termed NP-gamma (107). A fourth splicing variant of the PPT gene termed δ-PPT has also been identified in rat tissues, which predicts the existence of a novel tachykinin NP-δ (108). NKB is coded by a different gene.

Localization

SP is localized to sensory nerves in the airways of several species, including humans (2, 107, 109), although there has been debate about whether SP can be demonstrated in human airways (110). Rapid enzymatic degradation of SP in airways and the fact that SP concentrations may decrease with age and possibly after cigarette smoking could explain the difficulty in demonstrating this peptide in some studies. SP-IR nerves in the airway are found beneath and within the airway epithelium, around blood vessels, and, to a lesser extent, within airway smooth muscle. SP-IR nerve fibers innervate parasympathetic ganglia, suggesting a sensory input that may modulate ganglionic transmission and so result in ganglionic reflexes. In the nose, tachykinin containing nerve fibers are present in highest density in arterial vessel walls but are also present in venous vessels, gland acini, and in the epithelium (111) (figure 4).

SP appears to be localized predominantly to capsaicin-sensitive unmyelinated nerves in the airways. SP is predominantly synthesized in the nodose ganglion of the vagus nerve and then transported down the vagus to peripheral branches in the lung. Some SP-IR nerves also arise in dorsal root ganglia (112), but whether this proportion of nerves has a similar distribution and function as those arising from the nodose ganglion is not certain. Treatment of animals with capsaicin, bradykinin, histamine, the nicotinic agonist dimethylphenyl piperazinium, and electric nerve stimulation causes acute SP, NKA, and CGRP release from sensory nerves in the lung and heart (14, 113). Chronic administration of capsaicin only partially depletes the lung of tachykinins and CGRP indicating the presence of a population of capsaicin-resistant SP-IR nerves as in the gastrointestinal tract (14). Capsaicin acts upon a nonselective cation channel to induce acute sensory nerve depolarization (114). Chronic or high-dose exposure leads to chronic calcium leak into cells with subsequent inactivation of calcium-dependent enzymes and osmotic lysis (114). NKA-IR has been demonstrated in human airways and appears to be colocalized with SP (115). NPK is also present in the airways, whereas NKB does not appear to be present (115). It is not certain whether NP-gamma or NP-δ are present.

Receptors

Tachykinin effects on target cells are mediated via specific receptors and each tachykinin appears to selectively activate a distinct receptor: NK_1 receptors are activated preferentially by SP, NK_2 receptors by NKA, and NK_3 receptors by NKB (116). Three distinct tachykinin receptors have now been cloned (117–120). A fourth tachykinin receptor has been suggested based on the potency of a series of synthetic tachykinin analogs in guinea-pig trachea (121). With the development of selective agonists and antagonists, it has now been possible to differentiate subtypes of NK_2 receptor; thus the NK_2 receptor in tracheal smooth muscle appears to differ from that in urinary bladder and pulmonary artery (122, 123).

Autoradiographic studies have mapped the widespread distribution of SP receptors in guinea-pig and human lungs (124). SP receptors are found in high density in airway smooth muscle from trachea down to small bronchioles and vascular endothelium, whereas pulmonary vascu-

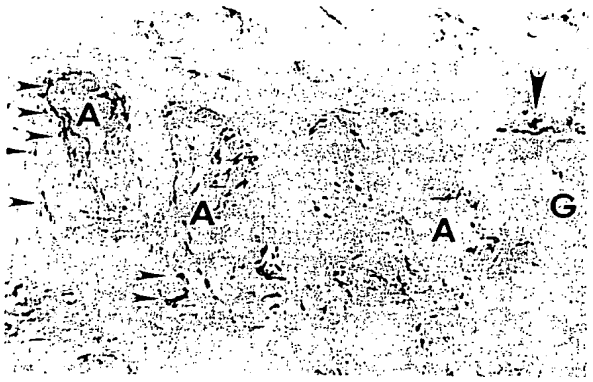

Fig. 4. Neurokinin A nerve fibers in human nasal mucosa. NKA-containing nerve fibers are seen as dark fibers in arterial walls (A) in human nasal mucosa. These vessels are extensively innervated. Representative fibers are indicated by arrowheads. A fiber (*large arrowhead*) is also seen near submucosal glands (G).

lar smooth muscle and epithelial cells are less densely labeled. Submucosal glands in human airways are also labeled. Similarly in human nasal mucosa, SP-binding sites are found in epithelium, glands, and vessels (111). In contrast, NKA binding sites are present only on arterial vessels. Therefore, although SP and NKA are colocalized in the same nerves in the nasal mucosa and are likely to be released together from the same varicosities, their actions are likely to be different because of their receptor distributions.

Metabolism

Tachykinins are subject to degradation by at least two enzymes, angiotensin converting enzyme (ACE, EC 3.4.15.1, kininase I) and neutral endopeptidase (NEP, EC 3.4.24.11, enkephalinase) (125). ACE is predominantly localized to vascular endothelial cells and therefore breaks down intravascular peptides. ACE inhibitors, such as captopril, enhance bronchoconstriction caused by intravenous SP (126, 127) but not inhaled SP (128). NKA is not a good substrate for ACE, however. NEP appears to be the most important enzyme for the breakdown of tachykinins in tissues. Inhibition of NEP by phosphoramidin or thiorphan markedly potentiates the bronchoconstrictor effect of tachykinins *in vitro* (129–131) and after inhalation *in vivo* (128, 132). NEP inhibition also potentiates mucus secretion in response to tachykinins (133–135). NEP inhibition enhances e-NANC and capsaicin-induced bronchoconstriction caused by the release of tachykinins from airway sensory nerves (136, 137).

The activity of NEP in the airways appears to be an important factor in determining the effects of tachykinins; any factors that inhibit the enzyme or its expression may be associated with enhanced tachykinin effects. Reductions in NEP activity occur after respiratory tract infections (138–140), cigarette smoke (141), ozone (142), and high doses of toluene diisocyanate (143).

Another endopeptidase (EC 3.4.24.15) that effectively degrades tachykinins has also been described in rat airway epithelium and nerves (144). This enzyme is not inhibited by drugs that inhibit NEP, and its role in regulating tachykinin effects in the airway is not yet clear.

Effects on Airway Smooth Muscle

Although SP contracts airway smooth muscle of several species including humans (145, 146), NKA is considerably more potent (81, 115, 131, 147), indicating that an NK_2 receptor is likely to be involved. This is confirmed by the use of selective synthetic agonists that are resistant to enzymatic degradation. The NK_2-selective agonist [Nle^{10}]-NKA(4–10) is a potent constrictor of human bronchi *in vitro*, whereas NK_1- and NK_3-selective agonists are ineffective (122, 148, 149). An NK_2-selective antagonist, L659,877 is effective against NKA-induced bronchoconstriction in human bronchi *in vitro* (149). The NK_2-selective competitive antagonist R-396 appears to be 100 times more potent in hamster tracheal smooth muscle than the NK_2-antagonist MEN 10207, whereas the reverse is true in rabbit pulmonary artery, demonstrating that different subtypes of NK_2 receptor may exist (122). In guinea-pig trachea, bronchonconstriction is mediated by NK_1 as well as NK_2 receptors (149), and there is also evidence for an atypical "NK_4" receptor (121). The contractile response to NKA is significantly greater in smaller human bronchi than in more proximal airways, indicating that tachykinins may have a more important constrictor effect on more peripheral airways (150), whereas cholinergic constriction tends to be more pronounced in proximal airways. *In vivo* SP does not cause bronchoconstriction either by intravenous infusion (151, 152) or by inhalation (151, 153), whereas NKA causes bronchoconstriction after intravenous administration (152) and after inhalation in asthmatic subjects (153). Surprisingly the bronchoconstrictor effect of nebulized NKA in asthmatic patients is inhibited by prior treatment with cromolyn sodium, indicating that it is mediated indirectly, rather than via a direct effect on airway smooth muscle (154). Like most other spasmogens, tachykinins cause contraction of airway smooth muscle by stimulating phosphoinositide hydrolysis and increasing the formation of inositol (1, 4, 5) trisphosphate, which releases calcium ions from intracellular stores in airway smooth muscle (155). As expected NKA is more potent than SP in this respect.

Interaction with Epithelium

Airway epithelium modulates the bronchoconstrictor effect of many spasmogens, possibly via the release of a relaxant substance termed epithelium-derived relaxant factor (EpDRF), which may be similar but not identical to endothelium-derived relaxant factor (156, 157). This may be of functional relevance in asthma because airway epithelium is often shed, even in patients with relatively mild asthma (158). Mechanical removal of epithelium markedly potentiates the bronchoconstrictor effect of tachykinins (137, 155, 159–161). For NKA, the effect of epithelium removal can be mimicked by inhibiting neutral endopeptidase (NEP) with phosphoramidon (137). Because NEP is localized to airway epithelium, mechanical denudation may remove the major site of tachykinin metabolism. The situation for SP is more complex because SP may interact with NK_1 receptors on epithelial cells to release the putative EpDRF and other bronchodilators such as prostaglandin E_2 (PGE_2) (137). Epithelium removal also potentiates the effects of capsaicin, indicating that endogenous tachykinin effects are also enhanced (136, 137). If epithelium is shed in asthmatic airways any effects of tachykinins may be more pronounced, not only on airway smooth muscle, but also inflammatory effects of tachykinins in the mucosa and submucosa (figure 5).

Airway Secretion

SP stimulates mucus secretion from sub-

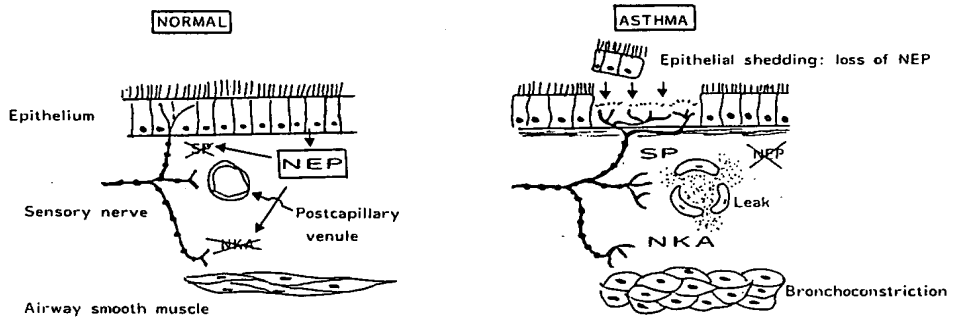

Fig. 5. Interaction of tachykinins with airway epithelium. When epithelium is intact neutral endopeptidase (NEP) degrades substance P (SP) and neurokinin A (NKA) released from sensory nerves (*left panel*). In asthmatic airways when epithelium is shed or NEP down-regulated any tachykinins released will have an exaggerated effect (*right panel*).

mucosal glands in animal and human airways *in vivo* (111, 133–135). In canine trachea, SP is one of the most potent stimulants of mucus secretion described, and at low concentrations appears to stimulate secretion without morphologic effects on secretory cells. This suggests that SP may cause the mycoepithelial cells that surround submucosal glands to contract and expel mucus from the glands and ducts rather like toothpaste is squeezed from a tube (162). Direct measurement gland duct secretion in cats confirms this suggestion (163). At higher concentrations, morphologic studies in ferrets suggest that SP stimulates serous cells (164). This is confirmed by functional studies that demonstrate an increased output of lysozyme, a serous cell marker (165). SP is more potent than NKA in stimulating airway mucus secretion, indicating that NK_1 receptors are involved (134, 165), and these have been localized by autoradiography to submucosal glands in human bronchi (124). SP stimulates glycoconjugate secretion without serous cell secretion from human nasal mucosa *in vitro* and as expected SP, but not NKA, binding sites are present in secretory cells in this tissue (111).

Stimulation of the vagus nerve causes discharge from goblet cells in guinea-pig trachea, as measured by a morphometric technique (166). This response is almost completely abolished by capsaicin pretreatment, indicating that the release of sensory neuropeptides is involved. Of the sensory neuropeptides, SP is by far the most potent in stimulating goblet cell secretion (167), indicating that an NK_1 receptor is involved. Because goblet cells are the only source of mucus in peripheral airways, it is possible that SP may play an important role in mucus secretion in peripheral airways in asthma and in cigarette smokers. Indeed cigarette smoke in guinea pigs results in marked goblet cell discharge, which is partly mediated by the vapor phase that activates capsaicin-sensitive nerves (168).

Tachykinins also stimulates ion transport in airway epithelium, with SP more potent than NKA (169), indicating that NK_1 receptors are involved. SP also releases PGE_2 and possibly EpDRF from airway epithelial cells (137, 159, 170). Tachykinins also increase mucociliary clearance in maxillary sinus (171) and in airways (172). In airways this response may be secondary to an increase in airway secretions.

Vascular Effects

Stimulation of the vagus nerve in rodents causes microvascular leakage, which is prevented by prior treatment with capsaicin or by a tachykinin antagonist, indicating that release of tachykinins from sensory nerves mediates this effect (173). Among the tachykinins, SP is most potent at causing leakage in guinea-pig airways (174) and NK_1 receptors have been localized to postcapillary venules in the airway submucosa (175). The distribution of leakage after vagus nerve stimulation and after intravenous SP are similar, with maximal effect in the lower trachea and main bronchi, indicating a differential distribution of NK_1 receptors in postcapillary venules in different airways (176). Inhaled SP also causes microvascular leakage in guinea pigs, and its effect on the microvasculature is more marked than its effect on airway smooth muscle (177); inhaled SP causes an increase in airways resistance in anesthetized guinea pigs, but unlike the increased resistance seen after a cholinergic agonist, this is not reversed by a full inflation. Whether tachykinins cause microvascular leakage in human airways is not yet certain because no direct measurements have been made. Nevertheless SP causes a wheal in human skin when injected intradermally, which indicates the capacity to cause microvascular leak in human postcapillary venules; NKA is less potent indicating that an NK_1 receptor mediates this effect (178).

Tachykinins have potent effects on airway blood flow. Indeed the effect of tachykinins on airway blood flow may be the most important physiologic and pathophysiologic role of tachykinins in airways. In canine and porcine trachea both SP and NKA cause a marked increase in blood flow (56, 97, 179). Tachykinins also dilate canine bronchial vessels *in vitro*, probably via an endothelium-dependent mechanism (180). Tachykinins also regulate bronchial blood flow in pig; stimulation of the vagus nerve causes a vasodilatation mediated by the release of sensory neuropeptides, and it is likely that CGRP as well as tachykinins are involved (56). CGRP and VIP act as arterial dilators and have synergistic effects on SP-induced vascular permeability in skin (181).

SP and NKA increase nasal blood flow, with less effect on nasal airflow, suggesting an effect on resistance vessels such as arteriovenous anastamoses rather than on capacitance vessels (182). This effect is mimicked by neural stimulation and by capsaicin, indicating that tachykinin release may be an important endogenous mechanism. Nasal provocation with SP in subjects with allergic rhinitis produces a limited reduction in nasal airflow (160). Airflow through the rigid nasal cavities is determined by the thickness of the mucosal lining (183), which depends on the degree of filling of venous sinusoids. Nasal mucosal blood flow is regulated by sympathetic and parasympathetic influences and can be augmented by stimulation of sensory nerves.

Effects on Inflammatory Cells

Tachykinins may also interact with inflammatory and immune cells (184), although whether this is of pathophysiologic significance remains to be determined. SP degranulates certain types of mast cell, such as those in human skin, but this response is not mediated by a classic tachykinin receptor, because it is dependent on the N-terminal sequence of the peptide (185), whereas receptor binding is determined by the C-terminal sequence. In contrast, human lung mast cells do not degranulate in response to SP (186). In rats the bronchoconstrictor response to tachykinins is reduced by blocking the effect of 5-hydroxytryptamine released from mast cells (rather than histamine), and also by cromolyn sodium and ketotifen, which may act by stabilizing mast cells (187). Furthermore, tachykinins increase the concentration of histamine in bronchoalveolar fluid of guinea pigs, indicating that they may directly activate airway mast cells (188). Surprisingly, NKA is more potent than SP in this respect, indicating that NK_2 receptors are involved (possibly on macrophages). Whereas similar measurements have not been made in human airways, indirect evidence that a similar phenomenon applies may be the demonstration that the bronchoconstrictor response to nebulized NKA in asthmatic patients is reduced by pretreatment with cromolyn sodium (154).

This raises questions about the functional innervation of mast cells in airways. Histologic studies have demonstrated a close proximity between mast cells and sensory nerves in airways (189). There is also evidence that antidromic stimulation of the vagus nerve leads to mast cell mediator release in canine airways (190). Furthermore, allergen exposure has effects on ion transport in guinea-pig airways that are dependent on capsaicin-sensitive nerves (191).

SP has a degranulating effect on eosinophils (192); again the degranulation is related to high concentrations of peptide and is dependent on the N-terminal sequence. Tachykinins have effects on

macrophage function *in vitro* and an NK_2 receptor appears to be involved (193). Tachykinins may activate monocytes to release inflammatory cytokines, such as interleukin-6 (IL-6) (194) and cause transient vascular adhesion of neutrophils in the airway circulation (195).

Effects on Nerves

In rabbits and ferrets, the bronchoconstrictor effect of tachykinins is partly mediated by the release of acetylcholine from postganglionic cholinergic nerves, because atropine reduces this response (196, 197). In guinea-pig trachea, tachykinins also potentiate cholinergic neurotransmission at postganglionic nerve terminals, and an NK_2 receptor appears to be involved (198). The potentiation is more marked at subthreshold voltages, suggesting that tachykinins may facilitate the *spread* of cholinergic transmission through postganglionic terminals, because this may increase the probability of neurotransmitter release from varicosities (199). Endogenous tachykinins may also facilitate cholinergic neurotransmission because capsaicin pretreatment results in a significant reduction in cholinergic neural responses both *in vitro* and *in vivo* (200, 201). Interestingly, capsaicin pretreatment also enhances i-NANC responses in airways, indicating that endogenous tachykinins may inhibit i-NANC mediated bronchodilatation (202). SP-IR nerves appear to innervate parasympathetic ganglia in airways, suggesting that endogenous tachykinins may also have a facilitatory effect on cholinergic neurotransmission at a ganglionic level. Indeed SP and capsaicin appear to enhance ganglionic neurotransmission (203). The interaction between tachykinins and human airway nerves is less certain. Although tachykinins do not facilitate cholinergic nerve-induced contraction of human bronchi under resting conditions, NKA has a facilitatory effect in the presence of potassium channel blockers (204).

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State of the Art

Neuropeptides in the Respiratory Tract

Part II[1-3]

PETER J. BARNES, JAMES N. BARANIUK, and MARIA G. BELVISI

Contents

Calcitonin Gene-related Peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide formed by the alternative splicing of the precursor mRNA coded by the calcitonin gene. There are two forms of CGRP which differ by three amino acids (205). Both α-CGRP and β-CGRP are expressed in sensory neurones (206), and both are potent vasodilators.

Localization

CGRP-immunoreactive (IR) nerves are abundant in the respiratory tract of several species. CGRP is costored and colocalized with substance P (SP) in afferent nerves (207, 208). CGRP has been extracted from and is localized to human airways and nasal tissue (209, 210). CGRP-containing nerve fibers appear to be more abundant than SP fibers, possibly because CGRP has greater stability, and is also present in some nerves that do not contain SP. CGRP is found in trigeminal, nodose-jugular, and dorsal root ganglia (211). Unlike SP it has also been detected in neuroendocrine cells of the lower airways (212).

Receptors

CGRP binds to specific surface receptors that are linked via a stimulating G-protein (G_s) to adenylyl cyclase, thus increasing intracellular cyclic AMP concentrations in vascular tissues (205). CGRP-receptors have been detected in lung by direct binding studies (213) and localized by autoradiographic mapping (214) (figure 6). At least two subtypes of receptor have been suggested on the basis of structure activity studies with CGRP analogs (215).

Vascular Effects

CGRP is a potent vasodilator, which has long-lasting effects. After intradermal injection in human skin, CGRP induces a long-lasting flare response (216). CGRP is an effective dilator of human pulmonary vessels *in vitro* and acts directly on receptors on vascular smooth muscle (217). It also potently dilates canine bronchial vessels *in vitro* (217) and produces a marked and long-lasting increase in airway blood flow in anesthetized dogs (218) and conscious sheep (219). Receptor mapping studies have demonstrated that CGRP-receptors are localized predominantly to bronchial vessels rather than to smooth muscle or epithelium in human airways (214) (figure 6). In human nasal tissues, CGRP binding sites are most dense on arterial vessels (210). It is possible that CGRP may be the predominant mediator of arterial vasodilatation in response to sensory nerve stimulation in the nose and bronchi* and so increases mucosal blood flow by acting on these resistance vessels. CGRP may be an important mediator of airway hyperemia in inflammation.

By contrast, CGRP has no direct effect of airway microvascular leak†. CGRP potentiates the leakage produced by SP in skin, presumably by increasing the blood delivery to the sites of plasma extravasation in the postcapillary venules (220). This does not occur in the airway when CGRP plus SP are administered intravenously, possibly because resting blood flow in the airways is higher†. It is possible that potentiation of leak may occur when the two peptides are released together from sensory nerves.

Effect on Airway Smooth Muscle

CGRP causes constriction of human bronchi *in vitro* (209). This is surprising because CGRP normally activates adenylyl cyclase, an event that is usually associated with bronchodilatation. Receptor mapping studies suggest few, if any, CGRP receptors over airway smooth muscle in human or guinea-pig airways, and this suggests that the paradoxical bronchoconstrictor response reported in human airways may be mediated indirectly, perhaps via release of bronchoconstrictor mediators. In guinea-

* Matran R, Alving K, Martling CR, Lacroix JS, Lundberg JM. Effects of neuropeptides and capsaicin on tracheobronchial blood flow in the pig. Acta Physiol Scand 1989; 135:335–42.

† Rogers DF, Belvisi MG, Aursudkij B, Evans TW, Barnes PJ. Effects of interactions of sensory neuropeptides on airway microvascular leakage in guinea pigs. Br J Pharmacol 1988; 95:1109–16.

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[3] This is Part II of two parts. Part I appeared in the last issue of the Review (Volume 144, No. 5).

pig airways, CGRP has no consistent effect on tone (221).

Other Airway Effects

CGRP has a weak inhibitory effect on cholinergically stimulated mucus secretion in ferret trachea (222) and on goblet cell discharge in guinea-pig airways‡. This is probably related to the low density of CGRP receptors on mucus secretory cells but does not preclude the possibility that CGRP might increase mucus secretion *in vivo* by increasing blood flow to submucosal glands. CGRP has no effect on human nasal mucosal secretion *in vitro* (210).

CGRP injection into human skin causes a persistent flare, but biopsies have revealed an infiltration of eosinophils (223). CGRP itself does not appear to be chemotactic for eosinophils, but proteolytic fragments of the peptide are active (224), suggesting that CGRP released into the tissues may lead to eosinophilic infiltration.

CGRP inhibits the proliferative response of T lymphocytes to mitogens, and specific receptors have been demonstrated on these cells (225). CGRP also inhibits macrophage secretion and the capacity of macrophages to activate T lymphocytes (226). This suggests that CGRP has potential antiinflammatory actions in the airways.

Neurogenic Inflammation

Pain (dolor), heat (calor), redness (rubor), and swelling (tumor) are the cardinal signs of inflammation. Sensory nerves may be involved in the generation of each of these signs. There is now considerable evidence that sensory nerves participate in inflammatory responses. This "neurogenic inflammation" is caused by the antidromic release of neuropeptides from nociceptive nerves or C-fibers via an axon reflex. The phenomenon is well documented in several organs including skin, eye, gastrointestinal tract, and bladder (227, 228). There is also increasing evidence that neurogenic inflammation occurs in the respiratory tract (229) and that it is possible that it may contribute to the inflammatory response in asthma (230).

Neurogenic Inflammation in Airway Disease

There are several lines of evidence that neurogenic inflammation may be important in airway disease.

Sensory neuropeptide effects. Sensory neuropeptides mimic many of the pathophysiologic features of asthma. Neurokinin A (NKA) is a very potent constrictor of human airways and enhances cholinergic neurotransmission; SP is a vasodilator, causes microvascular leakage, and stimulates mucus secre-

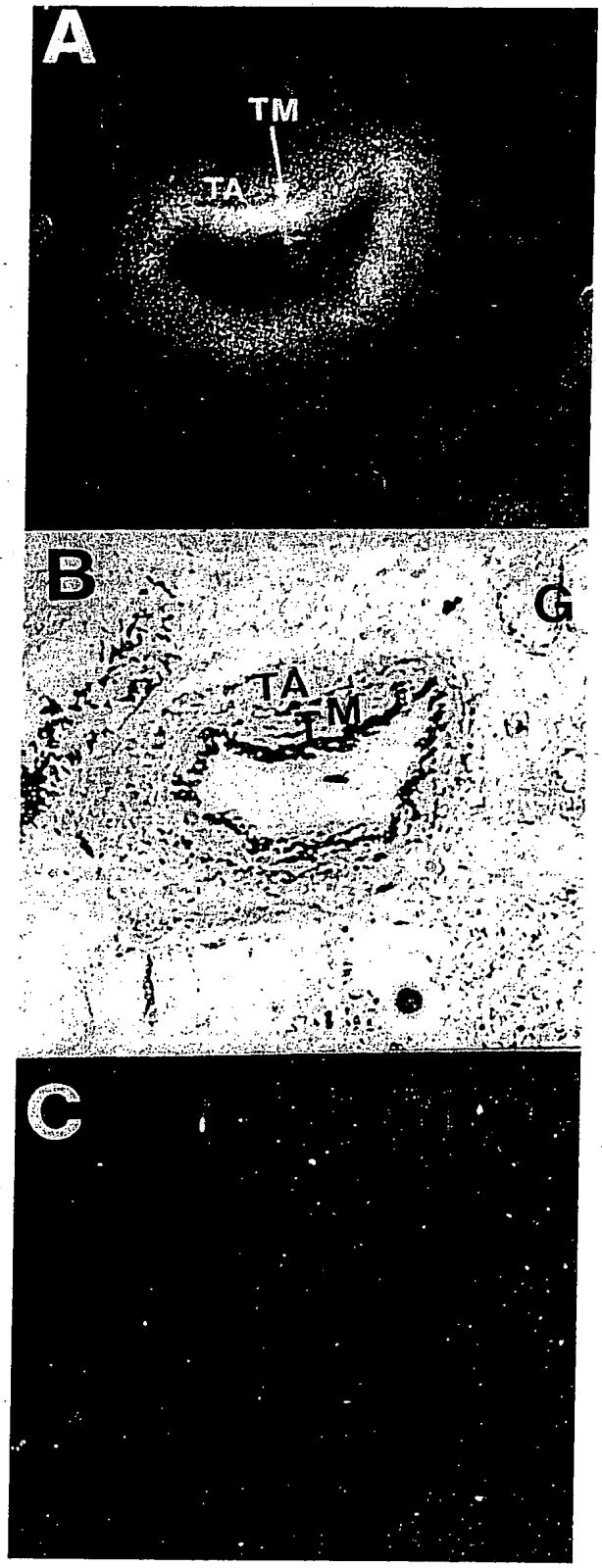

Fig. 6. Autoradiographic distribution of calcitonin gene-related peptide (CGRP) receptors in human airways. Panel A shows distribution of [^{125}I]CGRP binding to tunica media (TM) of a bronchial vessel but no labeling of endothelium or tunica adventitia (TA) (all gland). Panel B shows bright field view of the vessel. Panel C shows nonspecific binding in the presence of excess CGRP.

‡ Kuo H-P, Rhode JAL, Tokuyama K, Barnes PJ, Rogers DF. Capsaicin and sensory neuropeptide stimulation of goblet cell secretion in guinea pig trachea. J Physiol 1990; 431:629–41.

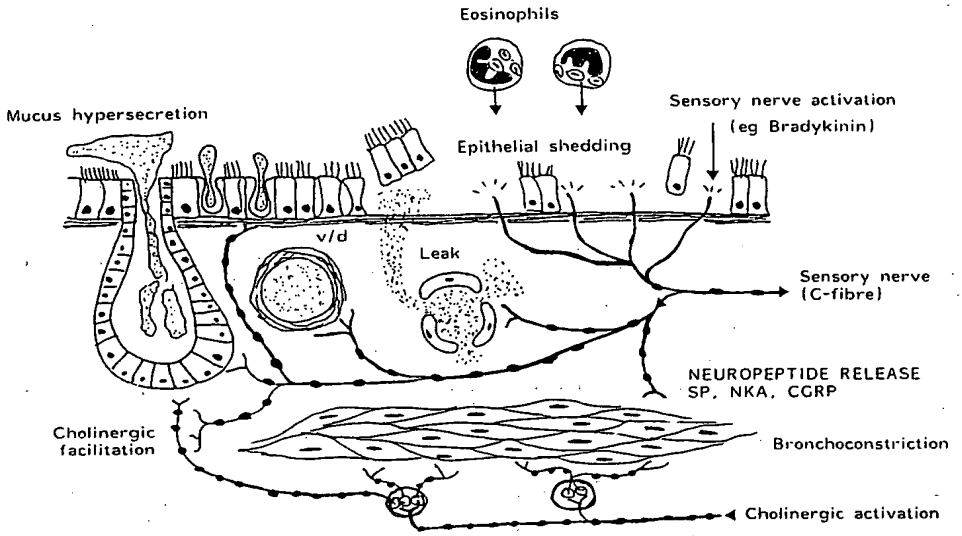

Fig. 7. Possible neurogenic inflammation in asthmatic airways via retrograde release of peptides from sensory nerves via an axon reflex. Substance P (SP) causes vasodilatation, plasma exudation, and mucus secretion, whereas neurokinin A (NKA) causes bronchoconstriction and enhanced cholinergic reflexes and calcitonin gene-related peptide (CGRP) vasodilatation.

tion from submucosal glands and epithelial goblet cells; CGRP is a potent and long-lasting vasodilator (figure 7). In addition, these peptides may have effects on regulation of local mucosal immunity.

Sensory nerve activation. Sensory nerves may be activated in airway disease. In cigarette smokers, components in the vapor phase of cigarette smoke, such as acrolein, may activate C-fiber endings (231, 232) leading to mucus hypersecretion (168). In asthmatic airways, the epithelium is often shed, thereby exposing sensory nerve endings. Sensory nerves in asthmatic airways may be "hyperalgesic" as a result of exposure to inflammatory mediators such as prostaglandins and certain cytokines. Hyperalgesic nerves may then be activated more readily by other mediators such as kinins.

Bradykinin is a potent bronchoconstrictor in asthmatic patients and also induces coughing and a sensation of chest tightness, which closely mimics a naturally occurring asthma attack (233). Yet it is a weak constrictor of human airways *in vitro*, suggesting that its potent constrictor effect is mediated indirectly. Bradykinin is a potent activator of bronchial C-fibers in dogs (234), and it releases sensory neuropeptides from perfused rodent lungs§. In guinea pigs, bradykinin instilled into the airways causes bronchoconstriction, which is reduced significantly by a cholinergic antagonist (as in patients with asthma [233]), and also by capsaicin pretreatment (235). This indicates that bradykinin activates sensory nerves in the airways and that part of the bronchoconstrictor response is mediated by release of constrictor peptides from capsaicin-sensitive nerves. Whether the bronchoconstrictor response to bradykinin seen in asthmatic patients is also caused by sensory peptide release is not certain, because specific tachykinin antagonists have not yet been studied in this situation. The inhibitory effects of cromolyn sodium and nedocromil sodium on bradykinin-induced bronchoconstriction provides supportive evidence (233, 236). Bradykinin nasal provocation induces vascular permeability and albumin secretion by direct actions upon vascular bradykinin receptors (237), and it stimulates pharyngeal discomfort by stimulating sensory nerves (238).

Pattern of innervation. Chronic inflammation may lead to changes in the pattern of innervation through the release of neurotrophic factors from inflammatory cells. Thus, in chronic arthritis and inflammatory bowel disease there is an increase in the density of SP-IR nerves (228, 239) and in NK_1-receptors (240). A striking increase in SP-IR nerves has been reported in the airway of patients with fatal asthma (241). This increased density of nerves is particularly noticeable in the submucosa. Whether this increase is caused by proliferation of sensory nerves or by increased synthesis of tachykinins has not yet been established. Cultured sensory neurons are stimulated by nerve growth factor (NGF), which markedly increases the gene transcription of β-prepotachykinin (β-PPT), the major precursor peptide for tachykinins (242). Because NGF may be released from several types of inflammatory cell, it is possible that this could lead to increased tachykinin synthesis and increased nerve growth. Several other neurotrophic factors have also recently been identified; however, bronchial biopsies of mild asthmatic patients have not revealed any evidence of increased SP-IR nerves. This may indicate that the increased innervation (241) may be a feature of either prolonged or severe asthma.

Neuropeptide metabolism. The metabolism of sensory neuropeptides may be impaired in asthmatic or chronic obstructive pulmonary disease airways. The activity of neutral endopeptidase (NEP) may be an important determinant of the extent of neurogenic inflammation in airways. Certain virus infections enhance excitatory nonadrenergic noncholinergic (e-NANC) responses in guinea pigs (243), and mycoplasma infection enhances neurogenic microvascular leakage in rats (244), an effect that is mediated by inhibition of NEP activity. Influenza virus infection of ferret trachea *in vitro* and of guinea pigs *in vivo* inhibits the activity of epithelial NEP and markedly enhances the bronchoconstrictor responses to tachykinins‖. Similarly, Sendai virus infection potentiates neurogenic inflammation in rat trachea#. This may explain why respiratory tract virus infections are so deleterious to patients with asthma. Hypertonic saline also impairs epithelial NEP function, leading to exaggerated tachykinin responses (245), and cigarette smoke exposure has a similar effect that can be explained by an oxidizing effect on the enzyme (246). Toluene diisocyanate, albeit at rather unrealistic doses, also reduces NEP activity and this may be a mechanism contributing to the airway hyperresponsiveness that may follow exposure to this chemical**.

§ Sária A, Martling CR, Yan Z, Theodorsson-Norheim E, Gamse R, Lundberg JM. Release of multiple tachykinins from capsaicin-sensitive nerves in the lung by bradykinin, histamine, dimethylphenylpiperainium, and vagal nerve stimulation. Am Rev Respir Dis 1988; 137:1330–5.

‖ Jacoby DB, Tamaoki J, Borson DB, Nadel JA. Influenza infection increases airway smooth muscle responsiveness to substance P in ferrets by decreasing enkephalinase. J Appl Physiol 1988; 64:2653–8.

Piedimonte G, Nadel JA, Umeno E, McDonald DM. Sendai virus infection potentiates neurogenic inflammation in rat trachea. J Appl Physiol 1990; 68:754–60.

** Sheppard D, Thompson JE, Scypinski L, Dusser DJ, Nadel JA, Borson DB. Toluene diisocyanate increases airway responsiveness to substance P and decreases airway and neutral endopeptidase. J Clin Invest 1988; 81:1111–5.

Thus, many of the agents that lead to exacerbations of asthma appear to reduce the activity of NEP at the airway surface, thus leading to exaggerated responses to tachykinins (and other peptides) and to increased airway inflammation. The role of NEP in human airway disease remains to be investigated.

Sensory nerve depletion. In several animal models of asthma, the role of neurogenic inflammation has been explored by selectively depleting sensory neuropeptides with capsaicin. In rat trachea, capsaicin pretreatment inhibits the microvascular leakage induced by irritant gases, such as cigarette smoke (247) and inhibits goblet cell discharge and microvascular leak induced by cigarette smoke in guinea pigs (248). Capsaicin pretreatment also reduces the vasodilator response to allergen in pig bronchi (249) and to toluene diisocyanate in rat airways (250). Capsaicin-sensitive nerves may also contribute to the bronchoconstrictor response to hypocapnia in rodents (251) but not to the acute bronchoconstrictor response to allergen (252). However, more prolonged exposure of sensitized guinea pigs to aerosolized antigen results in a pronounced increase in airway responsiveness, which is completely abolished by capsaicin pretreatment (253). This suggests that capsaicin-sensitive nerves may play an important role in chronic inflammatory responses to allergen.

Modulation of Neurogenic Inflammation

There are several ways in which neurogenic inflammation may be modulated (254) (figure 8), and these may provide novel approaches to antiinflammatory therapy in the future.

Inhibition of sensory neuropeptide effects. Antagonists of tachykinin or CGRP receptors should be effective. However, it has until recently proved difficult to develop antagonists that are potent and selective. An NK_2-receptor antagonist that has reasonable potency in human bronchi *in vitro* has been developed††, but this antagonist is a peptide and would therefore present problems with delivery. More potent tachykinin antagonists are now under development, and an NK_1-antagonist would be particularly useful because this receptor mediates most of the mucosal inflammatory effects of tachykinins. A potent nonpeptide NK_1-antagonist has recently been developed, and is likely to lead to a greater understanding of the role of SP (255). Because multiple peptides are released from sensory nerves (and there is no evidence for selective release), it is likely that antagonism of a single peptide may not be entirely effective. A more attractive approach may be to inhibit the release of all peptides.

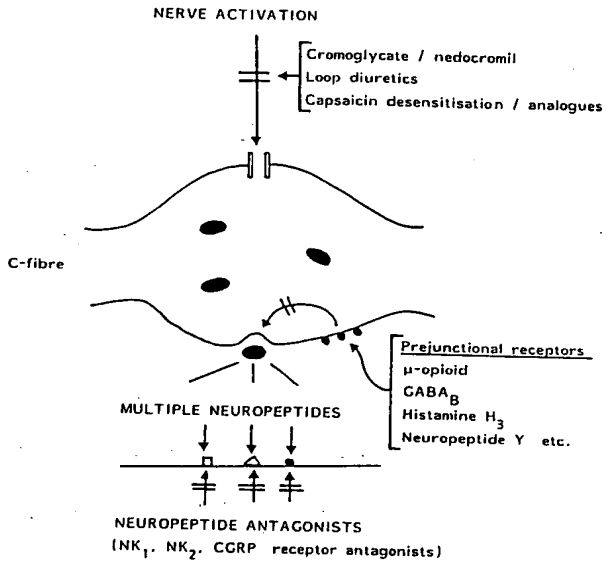

Fig. 8. Modulation of neurogenic inflammation. Neurogenic inflammation may be reduced by blocking the effects of neuropeptides released from sensory nerves (e.g., tachykinin antagonists) by inhibiting the release of peptides via activation of prejunctional receptors (e.g., μ-opioid receptors) or by inhibiting activation of sensory nerves (e.g., cromolyn sodium).

Inhibition of sensory neuropeptide release. Several different agonists act on prejunctional receptors on sensory nerves in airways to inhibit the release of sensory neuropeptides, thus inhibiting neurogenic inflammation. Opioids are the most effective agonists in this respect, and perhaps this is not surprising as they inhibit the release of SP from nociceptive fibers in the central nervous system (CNS) (256). Opioids inhibit e-NANC bronchoconstriction in guinea-pig bronchi *in vitro* (257–259) and *in vivo* (260, 261), while having no effect on equivalent tachykinin-induced bronchoconstriction, thereby indicating an effect on the release of tachykinins. This has now been confirmed because SP released from rat airways is potently inhibited by opioids (262). The effects of opioids are mediated via opioid receptors, because they are inhibited by naloxone. A μ-opioid receptor is involved since μ-selective agonists are effective, whereas δ- and κ-agonists are not (257, 259, 261). A μ-opioid agonist also modulates cholinergic neurotransmission in guinea-pig airways, but this is largely via an inhibitory effect on the facilitatory action of e-NANC nerves (259). Opioids also inhibit neurogenic microvascular leak in guinea-pig airways (260) (figure 8), mucus secretion from human bronchi *in vitro* (263), and cigarette smoke-induced goblet cell discharge from guinea-pig airways *in vivo* (248).

Several other agonists are also effective. These include the inhibitory central neurotransmitter gamma-amino butyric acid (GABA), which acts via a $GABA_B$-receptor (264), neuropeptide Y (265, 266), α_2-agonists (265, 267), galanin (268), corticotrophin releasing factor (269), β_2-agonists (270), adenosine via A_2-receptors (271, 272), and histamine via an H_3-receptor (273, 274). The fact that so many different receptors have an inhibitory effect raises the question of whether there is a common inhibitory mechanism. In the CNS, several of the agonists that are inhibitory to neuropeptide release have been found to open a common K^+ channel (275). The K^+-channel activator cromakalim and its active stereoisomer lemakalim are effective in inhibiting e-NANC bronchoconstriction in guinea pigs *in vivo* and *in vitro*. Their effects are inhibited by blockers of ATP-sensitive channels (276, 277). However, a potent ATP-sensitive channel blocker completely inhibits the effect of lemakalim but has no effect against the inhibitory actions of an opioid or α_2-agonist. By contrast, charybdotoxin, an inhibitor of large conductance Ca^{2+}-activated K^+ channels, is extremely effective in inhibiting these inhibitory actions, indicating that a common Ca^{2+}-activated K^+ channel may be involved that hyperpolarizes the sensory nerve and inhibits neuropeptide release.

Another agent that inhibits neuropep-

†† Rhoden KJ, Barnes PJ. Classification of tachykinin receptors on guinea pig and human airway smooth muscle (abstract). Am Rev Respir Dis 1990; 141:A726.

tide release from sensory nerves in airways is the red dye ruthenium red. Ruthenium red is a selective inhibitor of capsaicin-induced contraction of guinea-pig airways *in vitro* but has no effect against e-NANC bronchoconstriction induced by electrical field stimulation or bradykinin (278). A synthetic analogue of capsaicin, capsazepine, is also a competitive blocker of capsaicin effects on peripheral sensory nerves but has no effect on electrically induced neuropeptide release (279).

Inhibition of sensory nerve activation. Activation of sensory nerves may be inhibited by local anesthetics, but it has proved to be very difficult to achieve adequate local anesthesia of the respiratory tract. Inhalation of local anesthetics, such as lidocaine, have not been found to have consistent inhibitory effects on various airway challenges and indeed may even promote bronchoconstriction in some patients with asthma (280). This paradoxic bronchoconstriction may be caused by the greater anesthesia of laryngeal afferents that are linked to a tonic nonadrenergic bronchodilator reflex (281).§§ Other drugs may inhibit the activation of airway sensory nerves. Cromolyn sodium and nedocromil sodium may have direct effects on airway C-fibers (282, 283), and this might contribute to their antiasthma effect. Nedocromil sodium is highly effective against bradykinin-induced and sulfur dioxide-induced bronchoconstriction in asthmatic patients (282, 284), which are believed to be mediated by activation of sensory nerves in the airways. In addition, nedocromil sodium, and to a much lesser extent cromolyn sodium, inhibit e-NANC bronchoconstriction in guinea-pig bronchi *in vitro*, indicating an effect on release of sensory neuropeptides as well as on activation (285). The loop diuretic furosemide given by nebulization, behaves in a similar fashion to nedocromil sodium and inhibits metabisulfite-induced bronchoconstriction in asthmatic patients (286) and also e-NANC and cholinergic bronchoconstriction in guinea-pig airways *in vitro* (287). In addition, nebulized furosemide also inhibits certain types of cough (288), providing further evidence for an effect on sensory nerves.

Replacement of NEP. Because defective function of NEP may be critical in amplifying neurogenic inflammation, another strategy would be to replace the enzyme. Indeed recombinant human NEP has been shown to inhibit cough induced by tachykinins in the guinea pig (289). It may also be possible to increase the activity of NEP in the airways. Thus, corticosteroids appear to increase the activity of NEP in airways, and this may be at the level of gene expression in epithelial cells (290). Perhaps one of the potential beneficial actions of inhaled corticosteroids in asthma is increased expression of NEP activity.

Sensory denervation. Exposure of adult animals to high concentrations of capsaicin depletes sensory neuropeptides, which are only slowly repleted. Topical application of capsaicin might therefore be a useful approach to controlling neurogenic inflammation in the respiratory tract. Although this may be difficult to achieve in the lower airways, it appears to be feasible in the nose. Nasal application of capsaicin is reported to be effective in controlling nonallergic vasomotor rhinitis for periods of over a year (291, 292).

Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide that is a cotransmitter with norepinephrine in adrenergic nerves and usually amplifies its effects (293).

Localization

The distribution of NPY follows the distribution of adrenergic nerves and is predominantly to nasal vessels and bronchial vessels and glands, with less marked innervation of airway smooth muscle (294, 295) ‖‖###. After extrinsic denervation in heart–lung transplantation recipients, there is an apparent increase in NPY-IR nerves, suggesting that there may normally be some descending inhibitory influence to the expression of this peptide (296). In rodents, depletion of sensory neuropeptides with capsaicin is associated with an increase in adrenergic nerves, indicating that there may be a reciprocal interaction between sensory and adrenergic innervation in lung (297). NPY may also be found within parasympathetic ganglia, where it coexists with VIP because sympathectomy does not completely deplete NPY. This suggests that there is a small population of NPY-IR fibers in the respiratory tract that are not sympathetic in origin.

Effects on Airway Tone

NPY has no direct effect on airway smooth muscle of the guinea pig (298) but may cause bronchoconstriction via release of prostaglandins (299). NPY has a modulatory effect on cholinergic transmission of postganglionic cholinergic nerves (298). This appears to be a direct effect on prejunctional NPY-receptors, rather than secondary to any effect on α-adrenoceptors. Prejunctional and postjunctional NPY receptors may differ, with prejunctional Y_2-receptors acting to inhibit adenylyl cyclase, whereas postjunctional Y_1-receptors stimulate phosphoinositide hydrolysis (300). NPY also has a modulatory effect on e-NANC bronchoconstriction both *in vitro* and *in vivo*, and this effect is surprisingly long lasting (265, 266).

Vascular Effects

NPY binding sites are present on arterial smooth muscle and arteriosinusoidal anastamoses in human nasal mucosa (295). These are also the sites of the highest densities of NPY nerve fibers. NPY is a potent vasoconstrictor in some vascular beds, acting predominantly on the resistance arterioles. NPY causes a long-lasting reduction in tracheal blood flow in anesthetized dogs (218), but it has no direct effect on canine bronchial vessels *in vitro* (180), suggesting a preferential effect on resistance vessels in the airway. NPY also causes long-lasting vasoconstriction in the nose and is released with sympathetic nerve stimulation, particularly at higher frequencies (301). NPY may constrict resistance vessels reducing mucosal blood flow and reducing microvascular leak through the reduction in the perfusion of permeable postcapillary venules. This mechanism has been demonstrated in the airways for epinephrine (302).

Effects on Secretion

NPY has no direct effect on secretion from ferret airways, although it has complex effects on stimulated secretion. NPY enhances both cholinergic and adrenergic stimulation of mucus secretion but inhibits stimulated serous cell secretion (98). NPY has no effect on mucin secretion from human nasal mucosal explants *in vitro* (295).

Gastrin Releasing Peptide

Gastrin releasing peptide (GRP) is a 27

§§ Lammers J-WJ, Minette P, McCusker M, Chung KF, Barnes PJ. Capsaicin-induced bronchodilation in mild asthmatic subjects: possible role of nonadrenergic inhibitory system. J Appl Physiol 1989; 67:856–61.

‖‖ Uddman R, Sundler F. Neuropeptides in the airways: a review. Am Rev Respir Dis 1987; 136(Suppl: 3–8).

Uddman R, Sundler F. Innervation of the upper airways. Clin Chest Med 1986; 7:201–9.

amino acid peptide and is the mammalian form of the amphibian peptide bombesin (303). Other shorter peptides that share the active C-terminal sequence have also been described. These peptides interact with specific receptors and initiate phosphoinositide metabolism and elevation of c-myc and c-fos mRNA levels (303, 304).

Localization

GRP/bombesin-IR is localized to neuroendocrine cells in human and animal lower airways (303, 305).*** Bombesin-IR peptides have been recovered from the bronchoalveolar lavage fluid of smokers (306). GRP-containing nerve fibers have been demonstrated around blood vessels and submucosal glands in the airways of several species (307). GRP has also been identified in trigeminal sensory nerves that innervate the nasal mucosa of humans and animals (308). The distribution of GRP-containing nerves in nasal arterial and venous vessels and glands is identical to that of SP, NKA, and CGRP (309). GRP/bombesin binding sites in nasal and bronchial epithelium are present on epithelial cells and submucosal glands (308, 310) (figure 9).

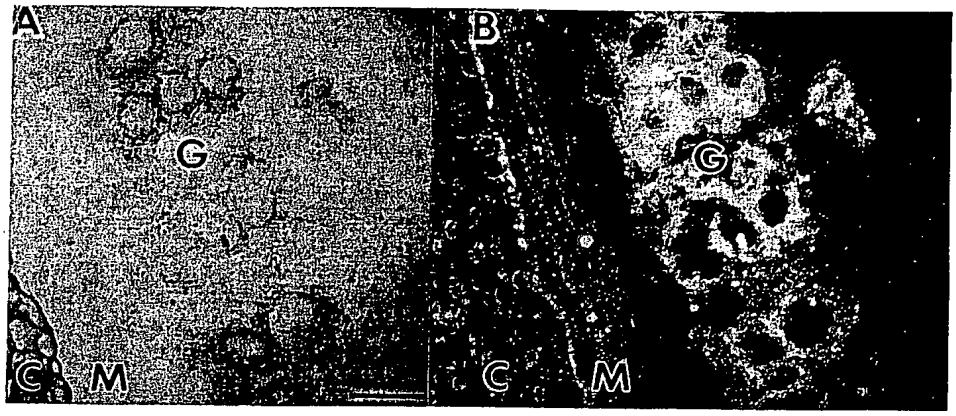

Fig. 9. Gastrin-releasing peptide (GRP) binding sites in human tracheal mucosa. *Left*: submucosal glands (G) and muscle (M) are seen. *Right*: darkfield view demonstrates white silver grains indicating [^{125}I]GRP binding sites in submucosal glands.

Airway Effects

GRP and bombesin-like peptides may play important roles in lung maturation. GRP mRNA production in lungs is increased on the day prior to birth and then declines (311). Bombesin-like immunoreactivity decreases with maturation (312). A marked reduction in bombesin-IR has been described in the lungs of infants who have died of fetal respiratory distress syndrome (312). Bombesin has a trophic effect on several cell types and may be important in epithelial growth (304, 313). Bombesin is secreted by certain small cell bronchial carcinomas and may have an autocrine effect on tumor growth (303).

Bombesin is a potent bronchoconstrictor in guinea pigs *in vivo* (314, 315). However, *in vitro* it has no effect on either proximal airways or on lung strips, indicating that it produces bronchoconstriction indirectly. The bronchoconstrictor response is not blocked by an antihistamine, cyclooxygenase inhibitor, lipoxygenase inhibitor, platelet activating factor antagonist, or serotonin antagonist, indicating that mediator release is unlikely; nor is it inhibited by capsaicin pretreatment or by cholinergic antagonists, suggesting that neural reflex mechanisms are not involved. The bronchoconstrictor response is inhibited by a bombesin receptor antagonist, BIM 26159, indicating that bombesin/GRP receptors are involved (315). Bombesin reduces tracheal blood flow in dogs, indicating a vasoconstrictor action (218).

GRP and bombesin are potent stimulants of airway mucus secretion in human and cat airways *in vitro* (308, 310) and guinea-pig nasal mucosa *in vivo* (316). GRP stimulates both serous cell lactoferrin and mucous glycoconjugate secretion from human nasal mucosa.

Other Peptides

Cholecystokinin

Cholecystokinin octapeptide (CCK_8) has been identified in low concentration in lungs and airways of several species†††. It may be localized to sensory nerves (317), although there are now suspicions that the visualized immunoreactivity reflects cross-reactivity with CGRP (318).

CCK_8 is a potent constrictor of guinea pig and human airways *in vitro* (319). The bronchoconstrictor response is potentiated by epithelial removal and by phosphoramidon, suggesting that it is degraded by epithelial NEP. The bronchoconstrictor effect of CCK_8 is also potentiated in guinea pigs sensitized and exposed to inhaled allergen, possibly because allergen exposure reduces epithelial NEP function. CCK_8 acts directly on airway smooth muscle and is potently inhibited by the specific CCK antagonist L363,851, indicating that CCK_A-receptors (peripheral type) are involved (319). CCK_8 has no apparent effect on cholinergic neurotransmission either at the level of parasympathetic ganglia or at postganglionic nerve terminals (319). Whereas few CCK-immunoreactive nerves are present in airways, it may still have a significant effect on airway tone if these particular neural fibers are activated selectively.

Somatostatin

Somatostatin has been localized to some afferent nerves (228), but the concentration detectable in lung is low. Somatostatin has no direct action on airway smooth muscle *in vitro* but appears to potentiate cholinergic neurotransmission in ferret airways (320). Although somatostatin has a modulatory effect on neurogenic inflammation in the rat foot pad (321), no modulation of e-NANC nerves in airways is apparent (Stretton CD, Barnes PJ, unpublished observations).

Galanin

Galanin is a 29 amino acid peptide named after its N-terminal glycine and C-terminal alanine (322). Galanin is widely distributed in the respiratory tract innervation of several species. It is colocalized with VIP in cholinergic nerves of airways and is present in parasympathetic ganglia (323) (20). It is also colocalized with SP/CGRP in sensory nerves and dorsal root, nodose and trigeminal ganglia†. Galanin has no direct effect on airway tone in guinea pigs but modulates e-NANC

*** Springall DR, Bloom SR, Polak, JM. Neural, endocrine and endothelial regulatory peptides. In: Crystal R, West JB, Barnes PJ, Cherniack N, Weibel ER, eds. The lung: scientific foundations. New York: Raven Press, 1991; 69-90.

††† Ghatei MA, Sheppard M, O'Shaunessy DJ, *et al.* Regulatory peptides in the mammalian respiratory tract. Endocrinology 1982; 111:1248-54.

neurotransmission (324). It has no effect on airway blood flow in dogs (218), and its physiologic role in airways remains a mystery.

Enkephalins

Leucine-enkephalin has been localized to neuroendocrine cells in airways (305), and [Met]enkephalin-Arg^6-Gly^7-Leu^8 IR nerves have been described in guinea-pig and rat lungs (317, 325), with a similar distribution to VIP (326). The anatomical origins and functional roles of the endogenous opioids is not clear because the opioid antagonist naloxone has no effect on neurally mediated airway effects (260, 261). However, it is possible that these opioid pathways may be selectively activated from brain stem centers under certain conditions. Exogenous opioids potently modulate neuropeptide release from sensory nerves in airways (257, 258, 260, 261) via μ-opioid receptors and may also modulate cholinergic neurotransmission via μ- or δ-opioid receptors (327).

Neurotensin

Neurotensin is a 13 amino acid peptide that was initially isolated from the hypothalamus but is also localized to epithelial cells and nerves in the gut (328). Its distribution in airways has not been reported. Neurotensin is a relatively potent constrictor of rat bronchi *in vitro*, and it also increases the contractile responses to cholinergic nerve stimulation, indicating that there may be facilitatory prejunctional receptors on airway cholinergic nerves in this species (329). Neurotensin also degranulates certain types of mast cell, although this may be related to its basic N-terminal sequence. Neurotensin is a substrate for NEP, and its bronchoconstrictor effects are potentiated by phosphoramidon in guinea pig (330). The effects of neurotensin on human airways have not yet been reported.

Conclusions

Many neuropeptides have now been localized to the respiratory tract, and certainly more will be discovered. These peptides often have potent actions on airway and vascular tone and on lung secretions, but the presence of so many peptides raises questions about their physiologic role. Unique combinations of peptides are colocalized and coreleased from the various subpopulations of sensory, parasympathetic, and sympathetic nerve fibers. The neuropeptides may produce synergistic and/or antagonist events at both pre and postsynaptic neurons, and on any surrounding target cells that possess the appropriate spectrum of peptide receptors. In this way, neuropeptides may act as subtle regulators of tissue activities under physiologic conditions. However, in inflammatory diseases such as asthma, they may have important pathogenetic roles. Alterations in degrading enzymes such as neutral endopeptidase may result in unopposed actions of proinflammatory neuropeptides. Until specific antagonists have been developed it will be difficult to evaluate the precise role of each of the neuropeptides in disease. It is certainly possible that pharmacologic agents that interact with neuropeptides by affecting their release, metabolism, or receptors may be developed in the future with therapeutic potential.

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Minireview

Tachykinins and tachykinin receptors: effects in the genitourinary tract

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Abstract

Tachykinins (TKs) are a family of peptides involved in the central and peripheral regulation of urogenital functions through the stimulation of TK NK_1, NK_2 and NK_3 receptors. At the urinary system level, TKs locally stimulate smooth muscle tone, ureteric peristalsis and bladder contractions, initiate neurogenic inflammation and trigger local and spinal reflexes aimed to maintain organ functions in emergency conditions. At the genital level, TKs are involved in smooth muscle contraction, in inflammation and in the modulation of steroid secretion by the testes and ovaries. TKs produce vasodilatation of maternal and fetal placental vascular beds and appear to be involved in reproductive function, stress-induced abortion, and pre-eclampsia. The current data suggest that the genitourinary tract is a primary site of action of the tachykininergic system.

Keywords: Tachykinins; Tachykinin receptors; Urinary tract; Genital tract

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Contents

Introduction

Tachykinins (TKs) are a family of peptides sharing a common C-terminal sequence (FXGLM-NH2) which enable them to act as full agonists of specific G-protein coupled receptors termed tachykinin NK_1, NK_2 and NK_3. Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are prototypic of endogenous NK_1, NK_2 and NK_3 receptor agonists, respectively; however the selectivity of endogenous TKs at these receptors is limited, so that the pharmacological characterization of TK receptors expressed on a given tissue requires the use of selective agonists or antagonists.

In the genitourinary tract, the major recognized sources of TKs are the primary afferent neurons expressing transient receptor potential vanilloid-1 receptors (TRPV1), which have the unique property of releasing transmitters both in the periphery (efferent function) and the spinal cord (afferent function) upon stimulation (Jancso et al., 1977; Lembeck and Holzer, 1979; see Lecci and Maggi, 2001, 2003, for reviews). These neurons typically express the alternative spliced beta-form of mRNA of the *TAC1* gene which codes for both SP and NKA. However, as will be detailed later, alternative non-neuronal sources such as neuroendocrine cells, uterus, placenta, and oocytes could become important in particular physiological conditions (Chiwakata et al., 1991; Pennefather et al., 1993, 2004; Page et al., 2000; Patak et al., 2003a; Pintado et al., 2003). Furthermore, expression of TKs in other neurons (capsaicin-resistant) could also potentially occur in pathophysiological conditions (e.g., inflammation); this has been demonstrated in airways (Lecci and Maggi, 2003) but as yet not in the genitourinary tract.

The importance of TKs expressed in sensory neurons in the regulation of lower urinary tract function is supported by experimental data on the co-localization of these peptides with TRPV1 receptors in various spinal cord segments. For instance, recently it has been shown that the co-

localization of TRPV1 and SP is prominent at L6 (to which urinary bladder afferents project) and decreases significantly at L4 (Hwang and Valtschanoff, 2003), thus reflecting the important role of spinal cord TK (NK_1) receptors in the regulation of the micturition reflex (Lecci and Maggi, 2001). Likewise, TKs affect genital function by acting at both central and peripheral levels (Dornan et al., 1993; Debeljuk and Lasaga, 1999; Debeljuk et al., 2003). TKs thus modify hypothalamic-pituitary-gonadal axis function in both males and females. These peptides modulate the secretion of gonadotropin-releasing hormone by the hypothalamus and of prolactin and gonadotropins by the anterior pituitary gland (Aronin et al., 1986; Debeljuk and Lasaga, 1999). Through an action on the hypothalamus TKs indirectly inhibit the secretion of luteinizing hormone (LH), although a direct action on the anterior pituitary to stimulate LH secretion has also been observed. Both actions have been proposed to be dependent on gonadal steroids. Orchidectomy in male animals and ovariectomy in females increase mRNA levels of *TAC3* (the gene that encodes NKB) in the arcuate nucleus, an effect similar to that reported in aging men with decreased gonadal function (Rance and Bruce, 1994; Danzer et al., 1999; Sandoval-Guzman et al., 2004). This increase in *TAC3* expression parallels that occurring in postmenopausal women (Rance and Young, 1991). The distribution and actions of SP, NKA, neuropeptide K (NPK) and neuropeptide γ (NPγ) on the hypothalamic-pituitary axis and pineal gland were recently reviewed (Debeljuk and Lasaga, 1999). In this review, we focus on the distribution and role of TKs and their receptors on the genitourinary tract at the peripheral level.

Tachykinins in the Lower Urinary Tract

Renal Pelvis and Ureter

A dense innervation of TRPV1-expressing afferent neurons containing TKs is present in the renal pelvis of various species, with fibers that are distributed within and just below the urothelium, in the muscle layer, and around blood vessels; whereas in the ureter the density of fibers that penetrate the urothelium is lower than in the upper tract. The urothelial content of TKs in humans is less than in animals, but the suburothelial plexus is still well represented; this location may enable the sensory nerves to detect a backflow of urine into the renal pelvis and ureteral wall and to activate both the afferent and efferent function of sensory nerves. TKs act as powerful stimulants of pyeloureteral motility by increasing the frequency and amplitude of spontaneous contractions and by increasing the basal tone (Santicioli and Maggi, 1998). In guinea pigs these effects could potentially involve the stimulation of both NK_1 and NK_2 receptors (Santicioli and Maggi, 1998) although NK_2 receptors play a pivotal role in the motor effects induced by endogenous tachykinins released by electrical stimuli (Patacchini et al., 1998). In rats the functional roles of NK_2 and NK_3 predominate over that of NK_1 receptors in terms of local effects (Maggi et al., 1988), although the latter receptors are important for the triggering of reno-renal reflexes (Kopp and Smith, 1993) and mediate cisplatin-induced nephrotoxicity (Alfieri and Cubeddu, 2000a). The expression of NK_3 receptors in the lower urinary tract is unusual, but this finding has been supported by the autoradiographic detection of NK_3 binding sites in the renal pelvis and proximal ureter (Chen and Hoover, 1995). In contrast, in both swine and human ureter, local excitatory effects induced by TKs are exclusively mediated by NK_2 receptors (Patacchini et al., 1998; Jerde et al., 1999). Interestingly, in the human ureter, unlike selective NK_1 or NK_3 antagonists, NK_2 receptor

antagonists also reduced spontaneous contractility, indicating that endogenous TKs may stimulate ureteral pacemaker activity through these receptors (Nakada et al., 2001). It is unknown, however, whether this stimulation is restricted to a subliminal inflammation linked to the experimental conditions or takes part in the physiological motility of this organ.

Urinary Bladder

As described for the ureter, the TK-expressing sensory neurons are distributed within and just below the urothelium, in the muscle layer, and around blood vessels; these fibers are more abundant in the bladder base than in the dome (Maggi, 1993). Immunohistochemical localization of TKs is almost identical with that of TPRV1 receptors in sensory (capsaicin-sensitive) nerve profiles, most of which also contain calcitonin gene-related peptide (CGRP) (Avelino et al., 2002), excitatory amino acids (glutamate and aspartate) (Keast and Stephensen, 2000), and pituitary adenylate cyclase activating peptide (PACAP). This latter peptide almost completely colocalizes with CGRP in capsaicin-sensitive afferent neurons in the bladder (Fahrenkrug and Hannibal, 1998). NK_1 receptors have been found in blood vessels and urothelium of all species thus far examined (Mussap et al., 1996; Burcher et al., 2000; Hammond et al., 2000), whereas their expression in muscle cells seems restricted to rats and guinea-pigs as was shown by autoradiographic studies and by the ability of selective agonists to induce contraction in isolated preparations (Nimmo et al., 1992; Maggi, 1993; Banasiak and Burcher, 1994; Lecci and Maggi, 2001). The function of NK_1 receptors in the urothelium and blood vessels seems intimately related to the inflammatory response, since NK_1 receptor antagonists inhibit inflammation-associated plasma leakage and the reduction of urothelial capacitance in a number of animal models (Hammond et al., 2000; Saban et al., 2000; Lecci and Maggi, 2001). In this context, it is likely that other cells involved in the inflammatory response, such as mast cells and neutrophils, also express NK_1 receptors, since selective antagonists or genetic ablation of NK_1 receptors reduce the number of neutrophils and associated bladder damage (Saban et al., 2000) by reducing the activity of inducible nitric oxide synthase (Alfieri and Cubeddu, 2000b). In the rat bladder, the activation of NK_1 receptors by SP also elicits reactive oxygen species generation, enhances intercellular adhesion molecule (ICAM) expression, and induces leukocyte infiltration and mast cell degranulation (Chien et al., 2003), further supporting the key role of these receptors in the induction of inflammation. Recently, it was shown that systemic SP administration induced the release of macrophage migration inhibitory factor, nerve growth factor, and the expression of cycloxygenase-2 and *c-fos* in bladder tissue, but the receptor(s) involved in these effects have not yet been characterized (Meyer-Siegler and Vera, 2004).

In bladder strip preparations, the stimulation of NK_1 receptors activates phospholipase C leading to inositol phosphate accumulation in rats and guinea-pigs, but not in hamsters (where these agonists are devoid of contractile activity), indicating that this response is linked to smooth muscle contraction (Suman-Chauhan et al., 1990; Torrens et al., 1995; Martin et al., 1997). Apart from direct contractile effects, the stimulation of NK_1 receptors enhances twitch contractions induced by electrical field stimulation (Fig. 1A) of efferent parasympathetic nerves mediated by acetylcholine and ATP on rat detrusor muscle. As evident from Fig. 1A, there are clear differences in the enhanced twitch response produced by septide and [Sar^9]SP-sulfone in that the maximal effect induced by the former agonist was larger than that of the latter (S. Meini, personal communication), whereas no differences in maximal effect were observed between these two agonists when their direct contractile activities were assayed on rat bladder strips (Meini et al., 1994). These NK_1 receptor agonists have also been reported to differ in

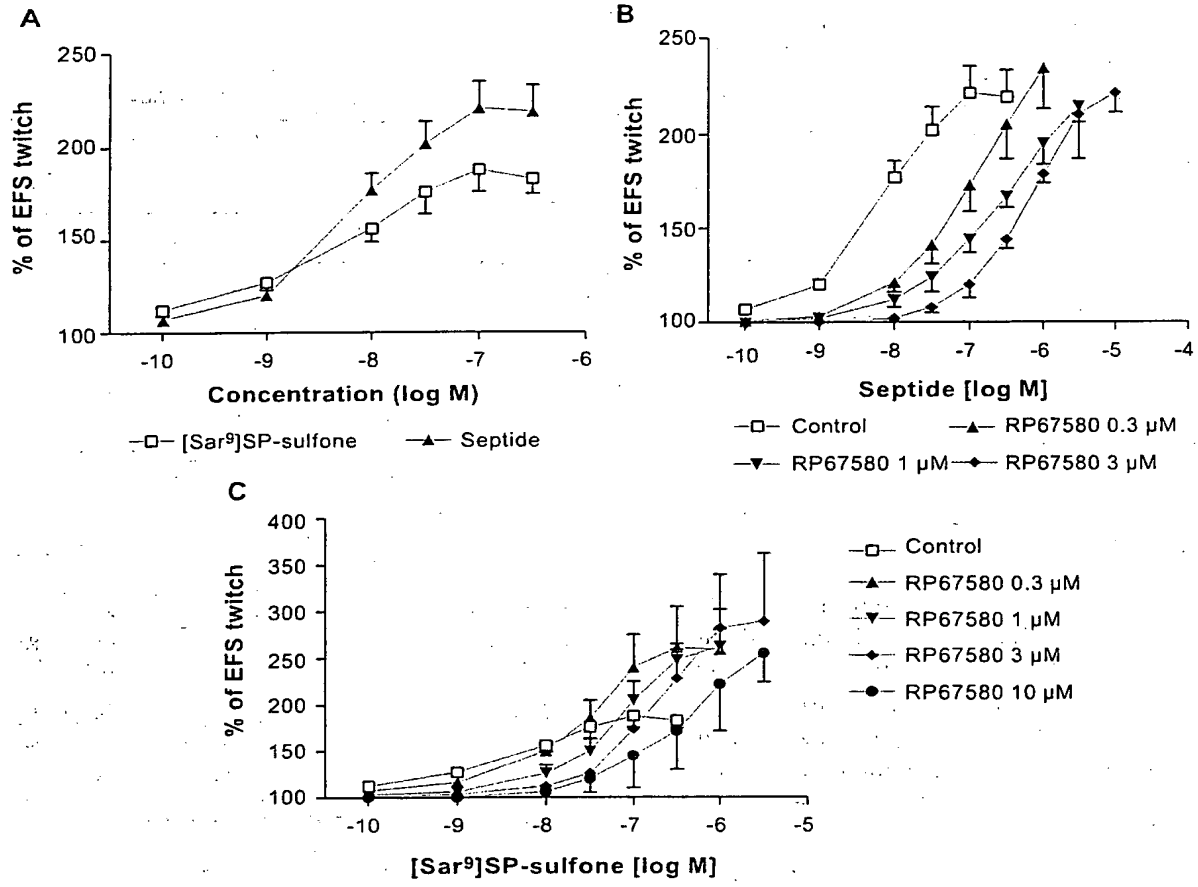

Fig. 1. (A) Mean log concentration-response curves for septide-(n = 9) and [Sar^9]SP-sulfone-(n = 12) induced facilitation of electrical field stimulation (EFS)-induced contractions in rat bladder. EFS parameters were: 5s trains every 30s, 2Hz, 60 V, 0.5ms pulse. (B) Mean log concentration-response curves for septide in the absence (n = 9) and in the presence of the selective NK_1 receptor antagonist RP67580 at 0.3 μM (n = 4), 1 μM (n = 4) and 3 μM (n = 4). (C) Mean log concentration-response curves for [Sar^9]SP-sulfone in the absence and presence of RP67580 at 0.3 μM (n = 6), 1 μM (n = 6), 3 μM (n = 5) and 10 μM (n = 3). Each bar represents the mean ± s.e. of mean for the given number of experiments. Data from S. Meini, unpublished.

their ability to stimulate cAMP formation, in which [Sar^9]SP-sulfone was much more effective than septide (Sagan et al., 1996), suggesting that the intrinsic activity in the potentiation of electrical field stimulation-induced contractions is inversely related to the activation of Gs. Interestingly, the selective NK_1 receptor antagonist RP67580 induced a concentration-related rightward shift of the septide effect (Fig. 1B). In contrast, when tested against [Sar^9]SP-sulfone, the antagonist increased the maximal effect of the agonist up to the level recorded with septide even at a concentration that did not produce any shift of the concentration-response curve of [Sar^9]SP-sulfone (Fig. 1C) (S. Meini, personal communication). These results could indicate that, unlike septide which only triggers excitatory responses, [Sar^9]SP-sulfone concomitantly activates excitatory and inhibitory mechanisms and that RP67580 would preferentially antagonize the latter ones.

When tested in vivo, peripherally-acting selective tachykinin NK_1 receptor antagonists inhibited SP-induced bladder hypermotility by blocking the generation of related reactive oxygen species (Chien et al., 2003). Nevertheless, this mechanism appears not to play a major role in models of inflammatory bladder hyperactivity since selective NK_1 receptor antagonists that do not penetrate into the CNS do not reduce this hyperactivity (Lecci and Maggi, 2001). Likewise, peripheral NK_1 receptors do not play a role in the micturition reflex induced by bladder distension in both rats (Lecci and Maggi, 2001) and guinea-pigs (Yamamoto et al., 2003). In contrast, a minor contribution of peripheral NK_1 receptors in mediating non-voiding contractions was reported in chronic spinal cord transected rats (Abdel-Gawad et al., 2001).

NK_2 receptors have been localized on detrusor muscle of all mammalian species studied (mouse, rat, guinea-pig, rabbit, dog, pig, sheep, monkey), including humans (Guard et al., 1993; Burcher et al., 2000; Tucci et al., 2001; Templeman et al., 2003b; see Lecci and Maggi, 2001 for a review) and in the suburothelial layer in rats (Nimmo et al., 1992; Carstairs et al., 1993; Lecci and Maggi, 2001). The stimulation of NK_2 receptors is coupled to inositol phosphate accumulation in rat, hamster (Suman-Chauhan et al., 1990; Torrens et al., 1995), and guinea-pig bladder (Martin et al., 1997). In the latter species NKA, unlike SP, also inhibited forskolin-triggered adenylate cyclase activity; however, since NKB was more potent than NKA in eliciting this effect, the type of TK receptor coupled to Gi remains uncertain (Martin et al., 1997). A further pathway that could be involved in TK receptor-mediated rat bladder contraction is the activation of Rho-kinase (Wibberley et al., 2003). In hamsters, urothelial prostanoid release contributes to NK_2 receptor-mediated bladder contraction both in vitro and in vivo (Tramontana et al., 2000), and a similar mechanism is also active in the rabbit detrusor (Sjogren et al., 1982), whereas blockade of cyclooxygenase failed to inhibit TK receptor-mediated contractile effects in rats, guinea-pigs, dogs and humans (Sjogren et al., 1982, Mussap et al., 1996; Warner et al., 2002). Although there is no morphological evidence for the expression of NK_2 receptors in urothelial cells, the fact that NK_2 receptor-mediated prostanoid release is almost abolished following urothelium removal (Tramontana et al., 2000) and that the stimulation of these receptors induces nitric oxide production in cultured rat urothelial cells (L.A. Birder, personal communication) provides strong evidence for this expression. In humans, NK_2 receptor-mediated bladder contraction largely depends on the activation of nifedipine-sensitive calcium channels (Maggi et al., 1989; Maggi, 1993; Lecci and Maggi, 2001).

The stimulation of NK_2 receptors enhances electrical field stimulation-induced twitch contractions in pig and rat bladder strips (Jiang et al., 1997); interestingly, this enhancement was selective on the purinergic component of the twitch (Templeman et al., 2003a). Because of their role during inflammation, it has been speculated that TKs could take part in the non-cholinergic component of the bladder contraction induced by electrical field stimulation, which is magnified in bladders from patients with detrusor instability. However, in detrusor specimens from patients affected by idiopathic detrusor instability, the atropine-resistant (but tetrodotoxin-sensitive) component of electrical field stimulation-induced contraction (that is enhanced in these patients) was not affected by the NK_2 receptor antagonist SR48968 (Moore et al., 2002), thus excluding the possibility that TKs enhance the efferent parasympathetic outflow in pathological conditions. However, it is worth noting that the modulation of electrical field stimulation-induced detrusor contractility by TK receptor antagonists can be appreciated only when stimulation parameters are set to recruit TRPV1 expressing sensory nerves. In these conditions, the late and the off component of the triphasic contraction are selectively reduced by NK_1 and NK_2 receptor antagonists, respectively (Meini and Maggi, 1994; Benko et al., 2003).

Electrophysiological experiments indicate that sensory neurons are not only the main bladder source of TKs but also an important target for urodynamic effects of TKs. In fact, NK_2 receptor

stimulation enhanced afferent nerve activity during physiological bladder distension without any increase of intravesical pressure (Kibble and Morrison, 1996a). This effect was blocked by a selective NK_2 receptor antagonist (SR48968), which was reduced by its own afferent discharge during bladder filling (Kibble and Morrison, 1996b). Recently it has been shown that TKs enhance the activity of N- and L-type calcium channels through the stimulation of NK_2 receptors in isolated small to medium size dorsal root ganglia neurons, providing further evidence for the neuronal expression of these receptors (Sculptorenau and De Groat, 2003). There is behavioral (Ruggieri et al., 2000), cystometrical (Lecci and Maggi, 2001), and immunohistochemical (Kiss et al., 2001) evidence that NK_2 receptors expressed on bladder afferent neurons are stimulated during bladder irritation, but they do not play an important role in signaling the bladder status under physiological conditions. Given these premises, it is not surprising that the blockade of NK_2 receptors reduces the amplitude and frequency of non-voiding contractions occurring during the filling phase of cystometry in chronically spinal cord-transected rats (Abdel-Gawad et al., 2001; Abdel-Karim et al., 2002). Not known, however, are the precise sites of action of the antagonists, i.e. whether muscular, neuronal, and/or urothelial NK_2 receptors could be potentially involved in this effect.

It has been suggested that muscular and neuronal NK_2 receptors can be distinguished by pharmacological analysis (Carstairs et al., 1993), and the recent identification of a NK_2 receptor mRNA splice variant (defined as $NK_2\beta$ receptor) in the rat urinary bladder further supports this hypothesis (Candenas et al., 2002). However, NKA and other TKs neither increased intracellular calcium concentrations and inositol phosphate production nor bound to $NK_2\beta$ receptors expressed on CHO cells, suggesting that this receptor isoform is nonfunctional (Bellucci et al., 2004).

At the peripheral level, NK_3 receptor mRNA has been detected in the rat urinary bladder but not in dorsal root ganglia (McCarson, 1999), which agrees with the lack of effect of a selective antagonist on pelvic nerve afferent activity induced by bladder distension (Julia et al., 1999) and with the autoradiographic localization of putative NK_3 receptors on postganglionic fibers (Nimmo et al., 1992). However, NK_3 receptor-mediated inhibition of N-type calcium channels has been recently described in isolated rat dorsal root ganglia (Sculptorenau and De Groat, 2003). In the urinary bladder, the expression and functional role of these receptors are controversial issues since some authors have found motility changes upon local application of selective agonists, whereas others have not (see Lecci and Maggi, 2001 for a review).

Urethra

Tachykinin receptors have been characterized in the urethra by means of in vitro contractility studies. In the rat proximal urethra both selective NK_1 and NK_2 receptor stimulation induced contractions, although the maximal effects induced by NK_2 receptor agonists were larger than those by NK_1 agonists (Maggi et al., 1988). In guinea-pigs, TK-induced proximal urethra contraction is exclusively mediated through NK_1 receptor stimulation (Maggi and Patacchini, 1992), whereas in humans TK-induced urethral contraction is mediated by NK_2 but not NK_1 receptors (Parlani et al., 1990; Palea et al., 1996). Intra-arterial administration of SP induced a phasic urethral contraction in female rats (Radziszewski et al., 2003); however peripheral TKs are not involved in the modulation of urethral motility during micturition since administration of selective NK_1 or NK_2 receptor antagonists did not change urethral basal tone or reflex motor activity in rats, cats, or guinea-pigs (Doi et al., 1999; Lecci and Maggi, 2001; Kamo and Doi, 2001). Although the contribution of tachykinins to urethral motility in pathophysio-

logical models is uncertain, there is evidence of a prominent NK_1 receptor-mediated inflammatory response at this level that is triggered by urethral irritation (Abelli et al., 1989; Abelli et al., 1991).

Tachykinins in the female reproductive tract

All presently known mammalian tachykinins, their precursor genes, and TK-metabolizing enzymes are expressed in the female genital tract (Pinto et al., 1999; 2001; Patak et al., 2000a,b, 2002, 2003a; Pintado et al., 2003). SP- and NKA-like immunoreactivity is present in the capsaicin-sensitive afferent innervation of the genito-urinary tract in female mouse, rat, guinea-pig, human as well as other species so far examined (Ottesen and Fahrenkrug, 1990; Traurig and Papka, 1993; Papka and Shew, 1994; Patak et al., 2000a). Although several species and regional differences exist, SP-immunoreactive nerve terminals are generally associated with the myometrial and vascular smooth muscle and are distributed throughout the endocervix and near endometrial glands (Traurig et al., 1984, 1988; Samuelson et al., 1985; Heinrich et al., 1986; Ottesen and Fahrenkrug, 1990; Papka and Shew, 1994). TK mRNAs are also expressed in non-neuronal cells in uteri of non-pregnant female mice and humans, and in cumulus cells and oocytes from mice (Patak et al., 2003a; Pintado et al., 2003). NKB is expressed in human and rat placenta (Page et al., 2000), human, rat, and mouse uterus (Cintado et al., 2001; Patak et al., 2003a; Pintado et al., 2003), and in different types of reproductive cells of mice (Pintado et al., 2003). To date, the *Tac4* gene that encodes the newly discovered tachykinin hemokinin-1 has been shown to be present in mouse uteri as well as in oocytes and blastocysts from mice (Pintado et al., 2003). The mRNA of its human orthologs, endokinin B, is also expressed in the human uterus (Page et al., 2003).

Hormones and pregnancy regulate the expression of TKs, their degrading enzymes, and the receptors on which they act in the uterus (Casey et al., 1991; Ottlecz et al., 1991; Pinto et al., 1999; Hamlin et al., 2000; Patak et al., 2000a,b, 2003a,b; Shintani et al., 2000; Candenas et al., 2001; Crane et al., 2002). Reciprocally, TKs modulate the secretion of steroids by the testes and the ovaries (Debeljuk and Lasaga, 1999). Thus it is conceivable that TKs, in addition to subserving important functions as sensory transmitters, when released peripherally may affect functions of the female reproductive tract. Consistent with this view, antidromic stimulation of lumbosacral dorsal roots in rats causes capsaicin-sensitive plasma extravasation in the corpus uteri and cervix, indicating release of transmitters from C-fibres (Pinter and Szolcsanyi, 1995). In the same context, it has been shown that treatment of neonates with capsaicin causes a reduction in rodent fertility (Traurig et al., 1984, 1988; Pintado et al., 2003).

The actions of the mammalian TKs include enhancement of uterine contraction, dilatation of blood vessels in the uterine vasculature, release of cytokines from uterine mast cells, plasma extravasation (Grant et al., 2002; Collins et al., 2002), and regulation of electrolyte transport across the endometrium (Vetter and O'Grady, 1997; see Skrabanek and Powell, 1983; Traurig and Papka, 1993; Patak et al., 2000a for reviews). Effects on steroid secretion from bovine corpora lutea in vitro (Miyamoto et al., 1993) and contractility of the mammalian fallopian tube (Forman et al., 1985; Samuelson et al., 1985) have also been proposed. These actions suggest that TKs may play a role in regulating uterine and oviductal contractility (Patak et al., 2000a, 2003a), in cervical dilatation (Collins et al., 2002), in the symptoms of pre-eclampsia (Page et al., 2000), and in stress-induced abortion (Arck et al., 1995; Markert et al., 1997; Marx et al., 1999; Joachim et al., 2001).

The occurrence and proposed actions of SP on the female genito-urinary tract and in particular the uterus were reviewed in 1983 (Skrabanek and Powell, 1983) and 1993 (Traurig and Papka, 1993). More recently, the effects of a wider range of TKs have been reviewed (Patak et al., 2000a). In this section of the present review we focus on functional studies of the receptors mediating and the factors regulating the uterotonic effects of the TKs.

Effects on myometrial and oviductal contractility

Although several studies indicate that SP enhances oviductal contractility in the human (Forman et al., 1985; Samuelson et al., 1985; Gauwerky et al., 1989) the receptors mediating these effects have not been characterized. Autoradiographic studies with [^{125}I] Bolton-Hunter-labelled substance P (BH-SP) indicate the presence of NK_1 receptors in blood vessels of human fallopian tube tissue sections (Nimmo et al., 1989).

The majority of studies of TKs and their effects and their receptors on the mammalian uterus have been undertaken using the non-pregnant rat (Shew et al., 1991; Barr et al., 1991; Fisher et al., 1993; Pennefather et al., 1993; Fisher and Pennefather, 1997, 1998, 1999; Magraner et al., 1997, 1998; Moodley et al., 1999; Hamlin et al., 2000; Patak et al., 2000a; Shintani et al., 2000; Cintado et al., 2001; Pinto et al., 2001; Crane et al., 2002; Williams et al., 2003). SP, NKA, and NKB all cause concentration-dependent contraction of the longitudinally arranged myometrium from cycling (Moodley et al., 1999) and oestrogen-primed virgin rats (Pennefather et al., 1993; Magraner et al., 1998; Fisher and Pennefather, 1999). The NKA fragment NKA(4–10) which may be formed in vivo by peptidase activity is also uterotonic in this species (Fisher and Pennefather, 1998). Since uterine responses to applied mammalian TKs are constrained by degradation by peptidases, in particular neprilysin (Fisher et al., 1993; Pennefather et al., 1993; Fisher and Pennefather, 1997; Magraner et al., 1998; Moodley et al., 1999), the characterization of TK receptors mediating this effect by natural TKs must be carried out in the presence of peptidase inhibitors (Patak et al., 2000a). The relative potencies of mammalian TKs in causing contraction of the oestrogen primed rat uterus are NKA > SP = NKB (Pennefather et al., 1993; Fisher and Pennefather, 1997; 1999; Magraner et al., 1998). This order of potency is indicative of activation mainly of NK_2 receptors. Similar findings have been replicated in cycling rats, but the maximum responses to NKA in tissues taken at dioestrus and metoestrus were greater than those taken at proestrus and oestrus (Moodley et al., 1999). The presence of NK_2 receptor protein in rat uterus has also been established through radioligand binding studies (Barr et al., 1991; Pennefather et al., 1993).

The relative order of potencies of TK receptor selective agonists confirms the predominance of NK_2 receptors in the rat uterus. However, the fact that agonists selective for the NK_1, NK_2 and NK_3 receptors contract uterine preparations from pregnant, ovariectomized, untreated, and oestrogen-treated non-pregnant rats (Barr et al., 1991; Fisher et al., 1993; Fisher and Pennefather, 1997; Magraner et al., 1998; Hamlin et al., 2000; Candenas et al., 2001; Crane et al., 2002) suggests expression of all three known receptors in this species, an observation that was confirmed by molecular studies (Magraner et al., 1998; Pinto et al., 1999; Candenas et al., 2001). The uterus represents one of the few peripheral organs where both NKB and the NK_3 receptor have been detected.

It has been reported that oestrogen treatment down regulates responses mediated by NK_3 receptor-selective agonists (Pinto et al., 1999; Hamlin et al., 2000; Crane et al., 2002; Williams et al., 2003), while ovariectomy and age increase such responses (Pinto et al., 1999; Cintado et al., 2001). Molecular studies

have shown that the expression of *Tac2* and *Tacr3*, the genes that encode NKB and the NK_3 receptor, respectively, in the rat uterus are strongly repressed under conditions of oestrogen dominance (Pinto et al., 1999, 2001). These observations are consistent with those for the *Tac2* gene in the arcuate nucleus (Rance and Young, 1991) and argue for the existence of a link between oestrogen and the NKB/NK_3 receptor activation pathway. Ovarian steroids and/or pregnancy also appear to modulate the expression of *Tac1* and *Tacr1*, the genes that encode SP/NKA and the NK_1 receptor, in the female genital tract. However, there are conflicting reports regarding the effects of these hormones in NK_1 receptor function. In the rat uterus, oestrogen treatment down-regulated (Hamlin et al., 2000) or up-regulated (Pinto et al., 1999) contractile responses mediated by tachykinin NK_1 receptor selective agonists. The effects of oestrogen and progesterone on the expression of tachykinins and their receptors clearly merit further examination.

Selective TK receptor antagonists have proved useful in the characterization of TK receptors mediating uterine contraction in the rat. There is consensus that the NK_2 receptor selective antagonist SR 48968 inhibits the responses to NKA or selective NK_2 receptor agonists in both oestrogen-primed uterus and cycling rats (Fisher et al., 1993; Magraner et al., 1998; Fisher and Pennefather, 1999; Moodley et al., 1999). There is less agreement in the literature about the effects of SR 48968 on responses to SP and NKB in oestrogen-primed rats. Magraner et al. (1998) reported that its potency in antagonizing SP was approximately 100-fold less than that observed in inhibiting NKA, but was more effective in inhibiting NKB. In contrast Fisher and Pennefather (1999) reported that SR 48968 failed to antagonize the effects of SP and NKB, but they investigated only single, high concentrations of the latter peptides. In cycling rats, SR 48968 antagonized the effects of both NKA and NKB and to a lesser extent, SP (Moodley et al., 1999). In recent studies carried out on uteri from pregnant (Patak et al., 2002) and non-pregnant women (Patak et al., 2003a), the order of potency of natural TKs (NKA > SP = NKB), the observation that agonists selective for tachykinin NK_1 and NK_3 receptors were inactive (unlike the NK_2 receptor selective agonist [$Lys^5MeLeu^9Nle^{10}$]NKA(4–10)), and the effect of the NK_2 receptor selective antagonist SR 48968 (Patak et al., 2003a), converge to support the concept that NK_2 receptors predominate in mediating TK-induced human myometrial contraction.

The effects of the NK_1 receptor selective antagonist, SR 140333, on uterine responses to TKs have also been investigated. In oestrogen-primed rats it produced only a small antagonism of the effects of SP, despite being effective in inhibiting the responses to a selective NK_1 receptor agonist, [$Sar^9Met(O_2)^{11}$]SP (Magraner et al., 1998; Fisher and Pennefather, 1999). These results indicate that in the rat NK_1 receptors are coupled to uterine contraction but that most of the effects of SP on contractility are mediated by different receptors (possibly NK_2, see above). In contrast, in non-pregnant oestrogen-treated BalbC mice, SP-induced contraction is mediated exclusively through NK_1 receptor stimulation, whereas NK_2 receptors only play a minor accessory role in the response induced by NKA or NKB (Patak et al., 2002). The order of agonist potency (SP ≥ NKA > NKB) and the observation that the NK_2 and NK_3 receptor selective agonists [$Lys^5MeLeu^9Nle^{10}$]NKA(4–10) and [$MePhe^7$]NKB were relatively inactive indicate a prominent role for the NK_1 receptor. However, the NK_2 receptor was dominant in mediating the uterotonic effects of TKs in pregnant BalbC mice (Patak et al., 2003b; Candenas et al., 2003). This indicates that changes in the physiological status of the viscus, due to an altered hormonal environment, are capable of modifying the hierarchy of TK receptor types mediating uterine contractions. In addition, NK_1 receptors may participate in uterine functions other than in contractile responses. TKs cause oedema in the mouse uterus (Grant et al., 2002) and rat uterine cervix (Collins et al., 2002), the latter being mediated by the NK_1 receptor. It is

conceivable that, as occurs in many other peripheral organs, NK_1 receptors mediate pro-inflammatory effects of TKs in the female genital tract.

The effects of the NK_3 receptor antagonist SR 142801 on responses to some TKs and selective TK receptor agonists have also been investigated. This NK_3 receptor antagonist exhibits large species-dependent variations in affinity since it blocks guinea-pig NK_3 receptors in the nanomolar range while the affinity for the rat receptor is in the low micromolar range. Non-specific effects have also been detected at micromolar concentrations (Beaujouan et al., 1997). SR 142801 failed to inhibit NKB responses in cycling rats (Moodley et al., 1999) and those of mammalian TKs and selective agonists in oestrogen-primed rats (Magraner et al., 1998). Likewise, at a low nanomolar concentration, it exerted no blockade of senktide effects in tissues from oestrogen-primed rats (Fisher and Pennefather, 1997). However, at micromolar concentrations it partially inhibited the response to NKB in oestrogen-primed rats (Fisher and Pennefather, 1999) and the responses to NKA and [Nle^{10}]NKA(4–10), consistent with reports of some non-selectivity at this concentration in this species. In another study using tissue from ovariectomized rats, SR 142801 in a concentration-dependent manner inhibited the effects of senktide, with robust antagonism observed at submicromolar-micromolar concentrations (Hamlin et al., 2000). The discrepancy found in these studies (Fisher and Pennefather, 1997; Hamlin et al., 2000) might be attributed to the lack of oestrogen in the ovariectomized rats, the partial afferent denervation accompanying ovariectomy, or the use of different concentrations of SR 142801.

Only few studies on the actions of TKs on uterine preparations from pregnant rats have been published. It has been shown that the NK_2 receptor is the most important in mediating myometrial contractility during pregnancy in the rat (Candenas et al., 2001). In one study (Shintani et al., 2000) NKA was reported to be equipotent in increasing peak uterine force in preparations from non-pregnant rats of unknown oestrous cycle status and in preparations taken from rats on day 18 of pregnancy. However, in the presence of phosphoramidon to inhibit neprilysin, the potency of NKA was markedly increased in preparations from pregnant animals, but not from non-pregnant animals. These data are consistent with a reported up-regulation of neprilysin during pregnancy in the rat (Ottlecz et al., 1991). Based on reports that levels of NK_2 receptor mRNA remain mainly unchanged during pregnancy (Candenas et al., 2001), we may surmise that the enhanced potency of NKA in the pregnant animal observed by Shintani et al. (2000) might reflect more efficient receptor-effector coupling or increase in the Ca^{2+} sensitivity of the contractile machinery. Nevertheless it should be noted that the pD_2 values reported for NKA by Candenas et al. (2001) did not differ at any time during pregnancy from values for non-pregnant animals. In contrast to NKA, the contractile effects of selective agonists for NK_1 and NK_3 receptors were increased and decreased, respectively, at the end of pregnancy.

A recent study analyzed the relationships between myometrial content of SP and NK_1 receptors during the reproductive cycle in rats. SP-expressing afferent fibers, normally present in the myometrium, progressively disappeared during pregnancy (Schmidt et al., 2003). This effect was accompanied by an up-regulation of NK_1 receptors during the course of pregnancy, which reached a maximum at about the time of labor and decreased following delivery (Candenas et al., 2001; Collins et al., 2002; Schmidt et al., 2003). Changes in the expression of the NK_1 receptor during pregnancy were accompanied by parallel changes in NK_1 receptor-mediated functional responses (Candenas et al., 2001). Alm and Lundberg (1988) also reported the absence of SP- and NKA-like immunoreactivity in the late pregnant guinea-pig uterus, while no clear data are yet available for the human uterus. However, other studies

have shown no degeneration (Traurig et al., 1984) or even hypertrophy (Amira et al., 1995) of afferent innervation in the rat uterus during pregnancy.

Ovary

The presence of SP-immunoreactive nerve fibres distributed in different regions of the ovary has been established in different mammalian species (Dees et al., 1985; Barad et al., 1988; Kaleczyc et al., 1995; Majewski et al., 2002). In ovaries from juvenile and peripubertal rats, nerve fibers containing SP are found closely associated with the theca externa of antral follicles, in the interstitial tissue, and within the tunica adventitia of small blood vessels, mostly arterioles (Dees et al., 1985). Numerous studies have shown that tachykinins and their receptors are present in the ovaries of several mammalian species and modulate the secretion of steroids by these gonads (Ojeda et al., 1985; Angelova et al., 1991c; Pitzel et al., 1991; Miyamoto et al., 1993; Debeljuk, 2003). The concentration of SP, but not of NKA, in the rat ovary increases with age (Fernandez Alvarez et al., 2002). In the human ovary, thecal and stromal concentrations of immunoreactive SP-like activity were greater than those in the corpora lutea and tunica albuginea (Barad et al., 1988). Also, a recent report showed that the concentration of SP and NKA in the ovary of mice superovulated by treatment with equine pregnant mare serum gonadotropin and human chorionic gonadotropin was lower than in control, saline-treated animals (Debeljuk, 2003). Pintado et al. (2003) showed that cumulus cells isolated from superovulated mice express *Tac1*, *Tac2*, and *Tac4* mRNAs and low levels of *Tacr1* and *Tacr2*, while oocytes express *Tac1*, *Tac2*, and low levels of the three TK receptor mRNAs. There was also a significant expression of neprilysin, the main enzyme involved in tachykinin degradation at the uterine level. Further studies are needed to determine the physiological function of tachykinins in the ovaries.

Effects on blood flow

The powerful endothelium-dependent vasodilator effect of SP on uterine and ovarian vasculature has been studied in rabbits (Gram and Ottesen, 1982; Ottesen et al., 1983), guinea-pigs (Skrabanek and Powell, 1983; Fallgren et al., 1989), and humans (Stones et al., 1995), but there is a paucity of reports on its effects on uterine vasculature in rats and mice.

The presence of TKs and/or TK-like immunoreactivity in human placenta (Sastry et al., 1981; Skrabanek and Powell, 1983; Page et al., 2000) and in the sensory innervation of the female reproductive tract in the human has been reported (Forman et al., 1985; Barad et al., 1988; Stones et al., 1995; Butler-Manuel et al., 2002). As stated previously, TK-immunoreactive nerves are present in the adventitia of large blood vessels in the female genital tract (Samuelson et al., 1985; Heinrich et al., 1986). Several studies of TK action on human uterine vasculature showed that SP (Allen et al., 1988; Hansen et al., 1988a,b), and to a lesser extent NKB (Wareing et al., 2003), act as vasodilators. NKB is expressed and secreted from human and rat placenta and enters into both fetal and maternal circulations (Page et al., 2000). This tachykinin has been identified as a possible causative agent in pre-eclampsia (Page et al., 2000). During normal pregnancy, blood concentrations of NKB increase along gestational age and decrease following delivery (Sakamoto et al., 2003; Schlembach et al., 2003). In vitro studies have shown that NKB acts as a paracrine vasodilator in the human fetal placental circulation, which is mediated by an endothelium-independent direct effect on vascular smooth muscle cells through the

stimulation of NK_1 receptors (Brownbill et al., 2003; Laliberte et al., 2004). However, tachykinins produce pressor responses in both conscious and anesthetized animals and participate in the maintenance of hypertension in spontaneously hypertensive (but not normotensive) rats through stimulation of NK_3 receptors in the substantia nigra (Lessard et al., 2003). It is thus possible that, at least in certain pregnancies, NKB exerts a general vasoconstrictor effect while acting as a local placental vasodilator in order to maintain fetoplacental blood flow. In this same context, chronic administration of the selective tachykinin NK_3 antagonist SR 142801 causes a decrease of reproductive success and litter size in rats, again supporting a role for the NK_3 receptor in oocyte implantation and fetal trophism during physiological pregnancy (Pintado et al., 2003).

Tachykinins in the male reproductive tract

Primary sensory capsaicin-sensitive neurons expressing TK-like immunoreactivity supply the male genitalia, although some species and regional differences in their sources and distribution have been reported (Lange and Unger, 1990; Lakomy et al., 1997; Kaleczyc et al., 1999). Although TKs exert marked effects on the functions of most male genitalia and accessory glands, the receptors mediating their effects have to date been studied in only a few regions of the male reproductive tract. Nevertheless, it is clear that there are species differences in the distribution of the receptors mediating their typical stimulant effects in vas deferens and prostate (Lange and Unger, 1990; Palea et al., 1996; Buljubasich et al., 1999; Ventura et al., 2000).

Testis

Primary afferent spermatic neurons projecting from the dog testes exhibit SP-like and CGRP-like immunoreactivity (Tamura et al., 1996). SP-like immunoreactivity has also been reported in human, hamster, guinea-pig and mouse Leydig cells (Schulze et al., 1987; Angelova et al., 1991a; Chiwakata et al., 1991), but not rat or boar Leydig cells (Chiwakata et al., 1991). The *Tac1* gene is expressed in testicular Leydig and Sertoli cells (Debeljuk et al., 2003), but as yet there are no reports of the expression of *Tac2* or *Tac4* genes. Although the presence of SP in human sperm has been reported (Sastry et al., 1991), its source is not clear. In a recent study of tachykinin genes in mouse sperm, no mRNAs for tachykinin peptides were detected (Pintado et al., 2003). The presence of *βTac1* in the Siberian hamster testes indicates that SP, NKA and NPK are likely to be expressed in the testes in this species. TKs act indirectly on the testes through actions on the hypothalamus and the pituitary gland; they also have direct actions on isolated preparations of testis (Debeljuk et al., 2003). Thus SP, as well as NKA, NPK and NPγ, modify the release of testosterone from isolated Leydig cells (Angelova et al., 1991b). TKs have been shown to exert dual effects in enhancing the response of cultured hamster Leydig cells to luteinising hormone (LH) and to inhibit testosterone production (Kanchev et al., 1995; Angelova et al., 1996). TKs also enhance secretion of transferrin and lactate from cultured Sertoli cells (Rao et al., 1995; Debeljuk et al., 2003). Peptidases that degrade TKs are present in Sertoli cell cultures (Monsees et al., 1998), and these may affect relative peptide potencies. The order of potency of NPγ > NKA ≥ NPK > SP in enhancing the secretion of transferrin and lactate in cultured rat Sertoli cells (Rao et al., 1995) indicates an action at NK_2 receptors. In this context, several reports have shown the presence of mRNAs for both NK_1 and NK_2 receptors in human testes (Chiwakata

et al., 1991; Pinto et al., 2004) and a low expression of NK_2 receptor mRNA in mouse spermatozoa (Pintado et al., 2003). To date, however, no in vivo experiments indicating a clear function of these peptides within the testes have been reported.

Epididymis

The guinea-pig epididymis is supplied uniformly along its length with TK-immunoreactive nerve fibres (Greenberg et al., 1985). Rat epididymis represents a rich source of SP that stimulates sperm motility (Sastry et al., 1991). The human epididymis also expresses a dense SP-like immunoreactivity (Tainio, 1994). In the pig, the density of TK innervation of the cauda epididymis exceeds that of the caput epididymis (Lakomy et al., 1997). Despite this morphological evidence, there have been no reports to date on the functional response of smooth muscle of the cauda epididymis to TKs, although this tissue is responsive to other smooth muscle stimulants.

Vas deferens

In the pig, neurons expressing SP- and CGRP-immunoreactivity project predominantly ipsilaterally to the vas deferens from lumbar L2, L3, and sacral S2, and S3 pairs of dorsal root ganglia, with the lumbar neurons possessing the higher density (Kaleczyc et al., 2002). These neurons also project to the anterior pelvic (hypogastric) ganglion, indicating that the release of peptides from these collaterals could modify the activity of the efferent nerves supplying the vas deferens as well as influencing the tract more directly (Kaleczyc et al., 2002). The pattern of innervation of the vas deferens is similar in the mouse, rat, cat, guinea-pig, bovine and ovine vas deferens (Stjernquist et al., 1983; Takano et al., 1986; Geppetti et al., 1988; Ventura et al., 1998).

Capsaicin pretreatment of neonatal rats depletes the sensory neurons supplying the vas deferens of CGRP- and TK-like immunoreactivity (Ventura et al., 1998). Applied to isolated field stimulated preparations of vas deferens, capsaicin had dual effects in potentiating and inhibiting nerve-mediated twitch contractions of rat and guinea-pig vas deferens (Moritoki et al., 1987; Maggi et al., 1987; Ellis and Burnstock, 1989). In the mouse vas deferens the inhibitory effect is prominent (Parlani et al., 1995). The potentiation may reflect actions of released TKs, while the inhibitory effect is probably due to the release of CGRP (Saito et al., 1987; Santicioli et al., 1988).

TKs play an exclusively motor role in vas deferens of rat either directly contracting and/or potentiating neurally-mediated contractions (Fournier et al., 1980; Growcott et al., 1982; Lee et al., 1982; Hunter and Maggio, 1984; Kimura et al., 1984; Holzer-Petsche et al., 1985; Osakada et al., 1986; Rovero et al., 1989; McKnight et al., 1991; Miranda et al., 1991; Maggi et al., 1992b; Morimoto et al., 1992b; Williams et al., 1993; Hosoki et al., 1998). This is also the case for guinea-pig (Sjostrand and Swedin, 1968; von Euler and Hedqvist, 1974; Zetler and Kampmann, 1979; Growcott et al., 1982; Stjernquist et al., 1983; Rovero et al., 1989; Hall and Morton, 1991a,b; Patacchini et al., 1992; Anthes et al., 2002), mouse (Blackwell et al., 1978; Segawa et al., 1978; Parlani et al., 1995) and rabbit (Stjernquist et al., 1983; Maggi et al., 1992a).

Of the mammalian tachykinins, NKA is more potent than SP or NKB on rat vas deferens and produces contractions that are slower in onset and longer lasting than those to SP and NKB (Kimura et al., 1984; Holzer-Petsche et al., 1985), indicating actions at different receptors and/or different susceptibilities to inactivation. The relative potencies of mammalian TKs (Kimura et al., 1984;

Holzer-Petsche et al., 1985; Osakada et al., 1986), of non-mammalian TKs (Growcott et al., 1982; Lee et al., 1982) and of TK receptor-selective agonists (Tallon et al., 1993; Stavropoulos et al., 1995) and antagonists (Dion et al., 1990; McKnight et al., 1991; Fujii et al., 1992; Morimoto et al., 1992a,b; Hosoki et al., 1998), all indicate that the NK_2 receptor is prominent in mediating stimulant effects in the rat vas deferens. These functional observations are consistent with data from receptor binding experiments (Lee et al., 1986; Matuszek et al., 1998). Receptors for TKs on smooth muscle with a location distinct from those mediating neurotransmitter release have been proposed (Tousignant et al., 1987); the latter effect predominates at the prostatic end of the tissue. Both effects are mediated by an NK_2 receptor.

In contrast, the facilitation of neurotransmission to the smooth muscle of guinea-pig vas deferens is mediated by NK_1 receptors (Growcott et al., 1982; Hall and Morton, 1991b). Indeed, nerve stimulated preparations of guinea-pig vas deferens have been used successfully for screening of a number of novel agonists and antagonists of the NK_1 receptor (Dion et al., 1990; Hall and Morton, 1991b; Patacchini et al., 1992; Anthes et al., 2002). Radioligand binding studies suggest the presence of NK_1 receptors that may be associated with smooth muscle in the guinea-pig vas deferens (Mussap et al., 1989); a prejunctional site may also be involved.

Facilitation of sympathetic neurotransmission in mouse vas deferens may also be mediated by NK_1 rather than NK_2 receptors (Parlani et al., 1995). In contrast, NK_2 receptors are involved in the effects of TKs on rabbit vas deferens (Dion et al., 1987; Maggi et al., 1992a). Thus, there are clear species differences in the TK receptors mediating smooth muscle contraction in the vas deferens.

Seminal Vesicle

SP causes contractions and facilitates neurally mediated responses of seminal vesicle preparations of mouse, guinea-pig, and rabbit (Stjernquist et al., 1983). Although SP-immunoreactive nerves innervate seminal vesicles of most species (Gu et al., 1983; Stjernquist et al., 1983; Lange and Unger, 1990; Yuri, 1990; Sastry et al., 1991; Gosling and Dixon, 1994; Kaleczyc et al., 1999), very few studies on the effects of TKs on the smooth muscle of this organ have been conducted, and the nature of the TK receptor(s) mediating the effects of SP remain unknown.

Prostate

The literature indicates species differences in the roles and effects of TKs in the prostate gland (Lange and Unger, 1990; Palea et al., 1996; Buljubasich et al., 1999; Ventura et al., 2000). SP- and NKA-immunoreactivity in the rat and guinea-pig prostate is relatively sparse (Sastry et al., 1991). SP neither contracts smooth muscle nor facilitates neurotransmission to the rat prostate (Watts and Cohen, 1991; Buljubasich et al., 1999, Fig. 2). In contrast, both SP and NKA enhance electrically-evoked contractions of the guinea-pig prostate (Fig. 2). These effects are blocked by the NK_1 selective antagonist, SR 140333, but not by the NK_2 selective antagonist, SR 48968, indicating that TK effects are mediated by NK_1 receptors (Buljubasich et al., 1999; Ventura et al., 2000). Human prostate does not exhibit immunoreactivity for either SP or CGRP (Lange and Unger, 1990), but TKs cause contractions of the human prostate that are mediated by NK_2 receptors (Palea et al., 1996). In this context, the expression of *TAC1*, *TAC3*, and *TAC4* mRNAs in the human prostate has recently been described (Page et al., 2003; Pinto et al., 2004).

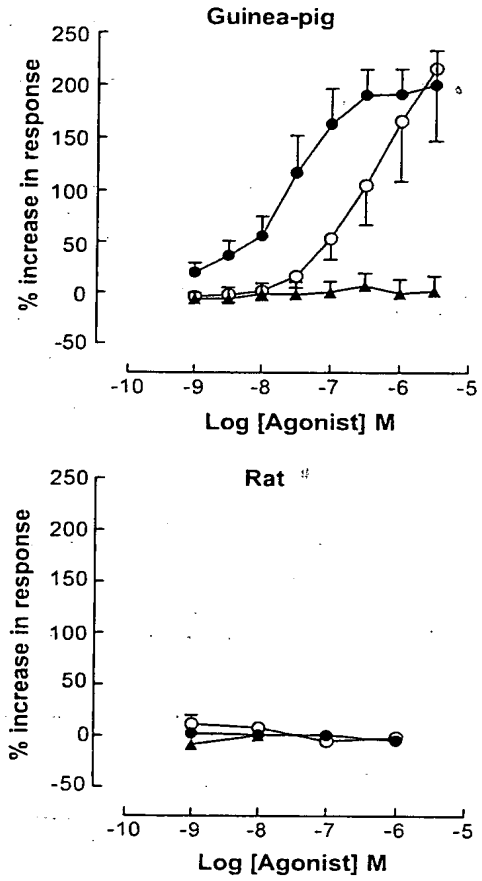

Fig. 2. Mean log concentration-response curves for tachykinin-induced facilitation of field stimulation-induced contractions in guinea-pig and rat prostate. Substance P (●), NKA (○) and senktide (▲). Each point is the mean ± s.e. mean of 4–9 observations. Data from Buljubasich et al. (1999).

Erectile tissue

SP is present in nerves innervating human erectile tissue and penile vessels (Hedlund and Andersson, 1985). It contracts human erectile tissue, is without effect on the cavernous artery, and partially relaxes noradrenaline-preconstricted preparations of corpus cavernosum and spongiosum (Hedlund and Andersson, 1985). In the human and rabbit corpus cavernosum the effects of SP are clearly endothelium-dependent (Azadzoi et al., 1992). NKA produces contractile effects of both corpus cavernosum and spongiosum that are mediated by NK_2 receptors (Patacchini et al., 2002). The selective NK_1 receptor agonist, [Sar^9]SP-sulfone, as well as septide, produced nitric oxide-mediated relaxations of high potassium-precontracted erectile tissues; these relaxations could be abolished by the NK_1 receptor selective antagonist SR 140333, further supporting the involvement of NK_1 receptors (Patacchini et al., 2002). In contrast, the stimulation of NK_1 receptors induces contractile responses in the rabbit corpus

cavernosum, and NK_2 receptors play a minor role in mediating the effects of natural TKs (Takahashi et al., 2002).

Blood vessels

The major innervation of blood vessels in the male reproductive tract is via sympathetic rather than sensory nerves. Antidromic stimulation of dorsal roots L3-L6 in rats (supplying the scrotum) does not produce plasma extravasation, indicating that the sensory neuropeptides in these nerves do not have a significant "efferent" effect on these blood vessels (Pinter and Szolcsanyi, 1995).

Autonomic ganglia supplying the male reproductive tract

NK_1 receptors are present in a majority of autonomic ganglia (Grkovic and Anderson, 1996), but many of the latter do not receive a dense supply of SP-immunoreactive nerves (Messenger et al., 1999). However, collaterals of TK-immunoreactive nerves supply the autonomic prevertebral ganglia innervating the pelvic viscera and parasympathetic ganglia, including the anterior pelvic ganglion in the male guinea-pig (Mitchell, 1993). SP can depolarize the neurons in ganglia (Minota et al., 1981; Dalsgaard et al., 1982; Dun and Jiang, 1982) and, with NKA, may be partly responsible for the slow excitatory synaptic potential induced by the stimulation of preganglionic fibers (Tsunoo et al., 1982; Saria et al., 1987). Thus it is possible that local autonomic reflexes involved in reproductive behavior may be initiated, at least in part, by release of TKs from collaterals of these sensory nerves.

Conclusions

Tachykinins, their receptors, and their degrading enzymes are widely distributed in the genitourinary tract, which appears to be a primary site of action of the tachykininergic system. In spite of clear species differences in the predominant TK receptor(s) mediating TK effects, these peptides play an important role in all species examined. At the urinary system level, TKs are not involved in normal motility but could be responsible for hypermotility, hypersensitivity, inflammation, and urothelial permeability changes that develop in pathophysiological models. In this context, NK_2 receptors play a pivotal role in the modulation of motor and sensory functions, whereas NK_1 receptors initiate inflammation and modulate immune function. However, we cannot exclude the possibility that tachykinin receptors could also be involved in the physiology of the urinary tract by regulating finer, yet unidentified processes. Current data also suggest that TKs may act as regulators of functions related to reproduction. TKs and the three known TK receptors are present at all main levels involved in the control of reproductive functions: the hypothalamus, the pituitary gland, the gonads, and the rest of reproductive tissues of both males and females. In both there is mutual regulation of tachykinin secretion by steroid hormones and of steroid secretion by tachykinins. In the female reproductive tract the expression of TKs and TK receptors, and of neprilysin, the enzyme primarily responsible for TK breakdown, are differentially modulated by gonadal hormones and pregnancy. Moreover, there is persuasive evidence that NKB plays a role as a mediator of pre-eclampsia symptoms. Future challenges will be to elucidate further the actions and roles of these peptides, particularly NKB and the NK_3 receptor, in reproductive function and to discover how the several products of the *TAC4* gene are involved in genitourinary functions.

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